Interleukin-6 and interleukin-10 are expressed in organs of normal young and old mice

Moshe Hacham[1], Rosalyn M. White[1], Shmuel Argov[2], Shraga Segal[1], Ron N. Apte[1]

[1] Department of Microbiology and Immunology, Faculty of Health Sciences, and the Cancer Center, Ben-Gurion University of the Negev, Beer-Sheva, Israel.
[2] Department of Pathology, Soroka Medical Center and Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel.

Correspondence: Ron N. Apte, Ph.D., Department of Microbiology and Immunology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel FAX: 972-8-6477-626 TEL: 972-8-6477-270.
E-mail: rapte@bgumail.bgu.ac.il

ABSTRACT. Interleukin-6 (IL-6) is a pleiotropic inflammatory cytokine, also endowed with inflammation-inhibiting properties. The status of interleukin-10 (IL-10) as an anti-inflammatory cytokine is more solidly established. The roles of IL-6 and IL-10 in the context of organ physiology, and their possible modulation by the aging process, are not satisfactorily understood. The purpose of this work was to characterize organ IL-6 and IL-10 expression in different cellular compartments in mice, under steady-state and stress conditions. The former was evaluated by immunohistochemistry (IHC) and enzyme-linked immunosorbent assay (ELISA) analyses of organ lysates (LYS) (addressing the intracellular compartment), while the latter was assessed by ELISA analyses of organ-conditioned media (CM), obtained after 48 hrs of organ culturing (addressing the potential of cytokine secretion/diffusion). Under steady-state conditions, the overall level of IL-6 and IL-10 expression was relatively low in both age groups (exceptionally, IHC staining demonstrated an enhanced expression of these cytokines in the heart, skeletal muscle and brain of young mice). Much more elevated levels of IL-6 and IL-10 expression were demonstrated in organ CM, possibly emphasizing the role of these cytokines in the context of organ stress. This was most characteristically shown in the highly specialized organs (heart, skeletal muscle and kidney) and liver of old mice, as compared with the other lymphoreticular organs (lungs, spleen, small intestine) tested. Thus, IL-10 was markedly upregulated in the highly specialized organs, while IL-6 was considerably reduced in the lymphoreticular organs. In addition, aging appears to be associated with altered patterns of intracellular expression and secretion/diffusion potentials of IL-6 and IL-10 in the heart and skeletal muscle, as demonstrated by reduced IHC staining on one hand, and an increased detection in organ CM, on the other. These findings may contribute to a better understanding of the unique functions of organ IL-6 and IL-10 in various age groups, and suggest an important role in organ response to stress in old age.

Keywords: IL-6, IL-10, cytokine organ expression, young/old age, homeostasis

Interleukin-6 is a pleiotropic, variably glycosylated cytokine involved in the regulation of immune responses and hematopoiesis [1-3]. IL-6 potentiates various immune/inflammatory functions, such as differentiation/proliferation or activity of B cells, macrophages, T cells and natural killer (NK) cells [1-3]. IL-6 also emerges as playing a dominant role in the elicitation of the acute phase response [4] and as an anti-inflammatory and immunosuppressive cytokine, including specific inhibition of interleukin-1 (IL-1) and tumor necrosis factor (TNF) synthesis and activity [2, 5]. IL-6 has also been described as influential in embryonic development and cardiogenesis [6] thumor growth modulation [7-9], downregulation of apoptosis/enhancement of cell survival [10-15], promotion of haemostasis [16] and organ protection against various destructive insults [17-19]. The specific activity of IL-6 is ultimately determined by the interactions of the IL-6 molecule with soluble IL-6 receptors (IL-6R) and gp130, and the distinct activation by these complexes of the cellular IL-6R/gp130 receptor system [20-21].

Interleukin-10 has been described so far as an inhibitory cytokine, produced by the Th2 subset of CD4 + helper cells, activated B cells, Th1 cells (in humans), activated macrophages and non-lymphocytic cell types, e.g. keratinocytes [22]. Its main immunosuppressive activity has been related to its ability to downregulate T-cell-mediated cellular immune inflammation through inhibition of various immune functions of the macrophage, and downregulation of proinflammatory cytokine expression/activity [22]. Recently, the spectrum of biological activities mediated by IL-10 has expanded to include antitumor effects [22], the induction of acute-phase proteins [22], inhibition of apoptosis [23-25] and stimulation of proliferation of different cells, such as keratinocytes, cytotoxic lymphocytes, NK and B cells [22, 63].

The physiological roles of IL-6 and IL-10 in organ homeostasis and defense are not fully understood. IL-6 expression has been widely detected in organs. In this context, we have shown that IL-6 expression is more pronounced in organs characterized by highly specialized

functions and increased vulnerability (heart, skeletal muscle, kidney and brain) [26]. The possible mediation by IL-6 of tissue-protective mechanisms operating under organ stress has been further substantiated in the heart and skeletal muscle [27-29].

IL-10 has been described in several tissues/organs, such as the heart, skin and mucosal epithelia, usually upon stimulation with pro-inflammatory agents or in disease [30, 31]. Its homeostatic role in the gastrointestinal tract, for example, is inferred from the emergence of severe gastrointestinal inflammation and colon cancer in IL-10-knock-out mice [32, 33]. Also, endogenous IL-10 may be cardioprotective [34].

An unambiguous concept of the roles played by organ IL-6 and IL-10 in old age physiology is lacking. Thus, apparently conflincting downregulation [35] and elevation [36] of IL-6 expression is reported with aging. Likewise, contrasting elevated or lowered IL-10 expression is depicted in old age [37, 38].

As the pleiotropic cytokines IL-6 and IL-10 are unique in their capability to both mediate and tightly regulate important defensive functions in the body, it was of interest to jointly assess their pattern of organ distribution in both young and old age groups. We used ELISA and immunohistochemistry to analyze the organ expression of these cytokines in different cellular compartments and under distinct conditions, aiming to better understand the physiological roles of IL-6 and IL-10 in organ homeostasis and under stress. This may be of special importance in old animals, as dysregulation in organ expression of these cytokines may pertain to various organ pathologies observed in senescence.

MATERIALS AND METHODS

Mice

C57BL/6 mice were bred under conventional, non-SPF-conditions at the animal facilities of the Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel. Eight-to-ten week-old female mice represented young mice, whereas 24-26 month (108-118 wks)-old female mice represented old mice. Routine periodical examination of the mice for common pathogens, performed in our animal facilities, including histological organ sections, showed no evidence of infection. Similarly, excised organs used in the experiments underwent thorough histopathological evaluation, which disclosed preserved tissue architecture and no signs of infection.

Recombinant cytokines and anti-cytokine antibodies

Murine rIL-6 and rIL-10, rat anti-mouse monoclonal antibodies to IL-6 and IL-10, and biotinylated rat anti-mouse monoclonal antibodies to IL-6 and IL-10 were purchased from Pharmingen, San Diego, CA, USA.

Analysis of IL-6 and IL-10 expression in organs

IL-6 and IL-10 organ expression was assessed by IHC in tissue sections and ELISA of organ LYS and CM obtained from organ culture.

The organ intracellular expression of these cytokines can be approximated at steady-state conditions through IHC staining (indicating the type of cytokine-producing cell and cytokine compartmentalization within it), and ELISA of tissue homogenates obtained immediately after organ excision. Quantitative cytokine assessment by ELISA of tissue homogenates (LYS) may thus reflect intracellular reservoirs of cytokines. Of note, due to potential differences in sensitivity of detection, these two methods may not necessarily be in complete correlation. On the other hand, through organ-culturing (CM), the secretion and possibly also the diffusion potential of IL-6 and IL-10 into the extracellular compartment can better be demonstrated, as demonstrated in many *in vitro*/*ex vivo* models, including under unstimulated conditions [39, 40].

Immunohistochemistry

The analyzed organs/tissues were fixed in formalin and embedded in paraffin. For immunohistochemical staining, organ sections (4 mm thick) were prepared, serially deparaffinized with xylene and then rehydrated with ethanol. After washing in phosphate-buffered saline (PBS), the sections were blocked for 1 hr by incubating in Cas-Blocker solution (0.5% w/v casein powder and 0.1% w/v sodium azide, in PBS, Sigma Chemical Company, St. Louis, MO, USA). Thereafter, the solution was stored at 4 ^{0}C, after its pH had been adjusted to 7.4. All incubations were performed in humidity chambers. Biotinylated rat anti-mouse monoclonal antibodies to IL-6 and IL-10 (primary antibodies at a concentration of 5 μg/ml) were then added in Cas-Blocker solution, followed by an overnight incubation at 4 °C.

Following extensive washings with 0.2% PBS-Triton-X-100 (3 consecutive, 15 min immersions), the endogenous peroxidase activity of sections was quenched with 9:1 methanol-3% H_2O_2 solution (slides immersed for 15 min). Thereafter, slides were washed in PBS-Triton-X-100 solution and reacted with the avidin-biotinylated horseraddish peroxidase complex (Elite/Vectastain ABC kit, Vector Laboratories) for 30 min. Following additional similar washings, the 3,3'-diamino-benzidine (DAB, Sigma) dye was added for 4 min. After stopping the dye reaction by washing in distilled water, the sections were counterstained with haematoxylin and mounted in Eukitt Mounting Medium (O. Kindler Gmb H,Co, Freiburg, Germany).

Omission of the specific primary antibody or its preincubation with excessive recombinant IL-6 or IL-10 (data not shown for the latter) resulted in abolition or significant diminution of the specific staining, respectively.

Preparation of organ-derived, conditioned media (CM) and lysates (LYS)

CM and LYS were prepared from organs of C57BL/6 mice, as described [40]. For the preparation of CM, the organs were removed on ice under aseptic conditions, rinsed three times in cold RPMI-1640, weighed and subsequently rinsed three times in a large volume of cold, antibiotic-containing (100 U/ml penicillin and 100 mg/ml streptomycin), serum-free RPMI-1640. Whole organs were then incubated for 48 hrs in identical RPMI-1640 medium at 37 °C. CM were then harvested, centrifuged (3000 rpm for 10 min) and sterilized by millipore filtration (0.45 mm, Corning Glass Works, Corning, NY, USA). Supernatants were then stored at – 20 °C before assay. For the preparation of LYS, organs were excised and

rinsed as previously mentioned. Organs were then cut into small pieces (approximately 3 mm^3) and subsequently homogenized on ice (0.1 g tissue /ml in RPMI-1640 containing 100 U/ml penicillin and 100 mg/ml streptomycin), using a homogenizer (Arthur H. Thomas Co., Philadelphia, PA, USA). The collected homogenate was cleared of debris by centrifugation (3000 rpm, 10 min) and LYS were sterilized, aliquoted and stored at – 20 ^{0}C.

To minimize the potential, non-physiological effects caused by the tissue proteases released under these conditions, lysates were prepared from freshly harvested organs/tissues, without additional incubation in cell-culture medium. Also, to neutralize the effects of tissue proteases in LYS and CM preparations, a mixture of protease/oxygenation inhibitors (phenylmethyl-sulfonyl fluoride (PMSF)-0.2 mM, aprotinin-10 mg/ml, leupeptine-10 mg/ml, pepstatin-10 mg/ml and dithiothreitol (DTT)-0.5 mM, all purchased from Sigma) was added during the preparation of organ samples. Standardization was achieved by diluting CM/LYS from the different organs in proportion to tissue weight. The arbitrary value of 0.1 g tissue/ml was chosen.

Protein level measurements in CM and LYS provided an additional criterion for standardization of the different samples. This was performed using the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). A mean value of 245 mg/ml, with negligible variation, was found in LYS from the different organs. Similarly, the average value of 90 mg/ml was found in CM.

IL-6 and IL-10 ELISA

ELISA aimed at detecting these cytokines in the organ samples, were performed based on a protocol suggested by the Pharmingen Company. The primary, cytokine-capture rat anti-mouse antibodies to IL-6 and IL-10 (2 µg/ml, diluted in 0.1 M $NaHCO_3$, pH 8.2, coating solution, Phamingen) were applied onto enhanced protein binding, 96-well ELISA plates and incubated overnight at 4 ^{0}C. After 2 washings with PBS/Tween, blocking was performed by 2 hrs incubation with PBS/10% FCS at room temperature. Following 2 washings with PBS/Tween, samples and standards were added at 100 ml/well for 1 hr (diluted in PBS/10% FCS). After intervening washings with PBS/Tween between the steps, biotinylated rat anti-mouse mAb to IL-6 and IL-10 (5 µg/ml, diluted in PBS/10% FCS serum) and the peroxidase conjugated streptavidin complex (1:100 diluted in PBS/10% FCS, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were sequentially applied for 45 and 60 min, respectively. Finally, after additional extensive washings with PBS/Tween (× 8), ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid Sigma) substrate solution was added. Absorbance was scored at a wavelength of 410 nm using the Dynateck MRX 5000 ELISA Reader (Dynex Technologies).

Statistical analysis of results

Each experiment was repeated 3-5 times with similar patterns of results. Thus, 3-5 separate tissue/organ preparations of the diverse experimental samples were used; each was obtained from a pool of 3-5 mice. ELISA were performed in triplicates: triplicate values in individual experiments did not differ from the mean by > 20%. The results obtained from the various experiments were pooled; shown are the means ± standard deviation. The statistical differences between the means representing the two age groups for individual organs were determined by a SPSS-11 statistical program (Anova test). One way Anova analysis served to determine the statistical differences between the means representing different organs in each age group. A value of $p < 0.05$ was considered as significant in both tests.

RESULTS

Altough obtained from experiments performed using in C57BL/6 mice, the results described here parallel and complement other findings on the issue of organ expression of IL-6 and IL-10, as elaborated below in other animal models or humans [6, 18, 27-30, 34, 35, 48, 58, 61, 62, 64]. Thus, they can be viewed in a broader perspective of organ expression of these cytokines and their relevance to body homeostasis and defense.

Organ expression of IL-6 and IL-10 as detected by immunohistochemistry

Relative elevated expression in highly specialized organs of young mice

As analyzed by immunohistochemistry, an enhanced level (relative to the lungs, small intestine, spleen and liver) of IL-6 and IL-10 was demonstrated in the heart, skeletal muscle and brain of young mice. The intensity of staining was reflected by the red-brownish colour, best demonstrated in the heart (B, *Figure 1* for IL-6 and *Figure 2* for IL-10). In the kidney, this pattern was detected only for IL-6 (A, *Figure 1*). IL-6 and IL-10 were detected mainly in the cytoplasm and nuclei of renal tubular cells (A) and diffusely, in the cardiomyocyte cytoplasm (B), as shown respectively for this group in *Figure 1* and *Figure 2*. For IL-10, the staining was also positive in cardiomyocyte nuclei *(Figure 2, B)*. An equivalent intensity of staining was demonstrated in the skeletal muscular myocytes and cerebral neurons (data not shown). In the first-line lymphoreticular organs, IL-6 and IL-10 expression was significantly lower. This is shown representatively in *Figure 1* (IL-6) and *Figure 2* (IL-10) for the spleen (C) and liver (D). In the spleen, weakly cytoplasm-stained, mononuclear cells were demonstrated rarely (*Figure 1, Figure 2*, C). Likewise, a similar weak staining was displayed in the nuclei and cytoplasm of perivenular hepatocytes *(Figure 2, Figure 3, D)*. Equivalent, low immunohistochemical staining was found in lumen-facing epithelial and mononuclear cells of the small intestine and lung bronchioles (data not shown). Of note, nuclear staining was evident in these epithelial cells when analyzing IL-10 expression (data not shown).

Decreased (highly specialized organs) and relatively unaltered (lymphoreticular organs) expression in old mice

In old mice, a decreased IL-6 expression (relative to young mice) was demonstrated in the kidney and heart *(Figure 1)*. A similar decreased staining was also found in the skeletal muscle and brain (data not shown).

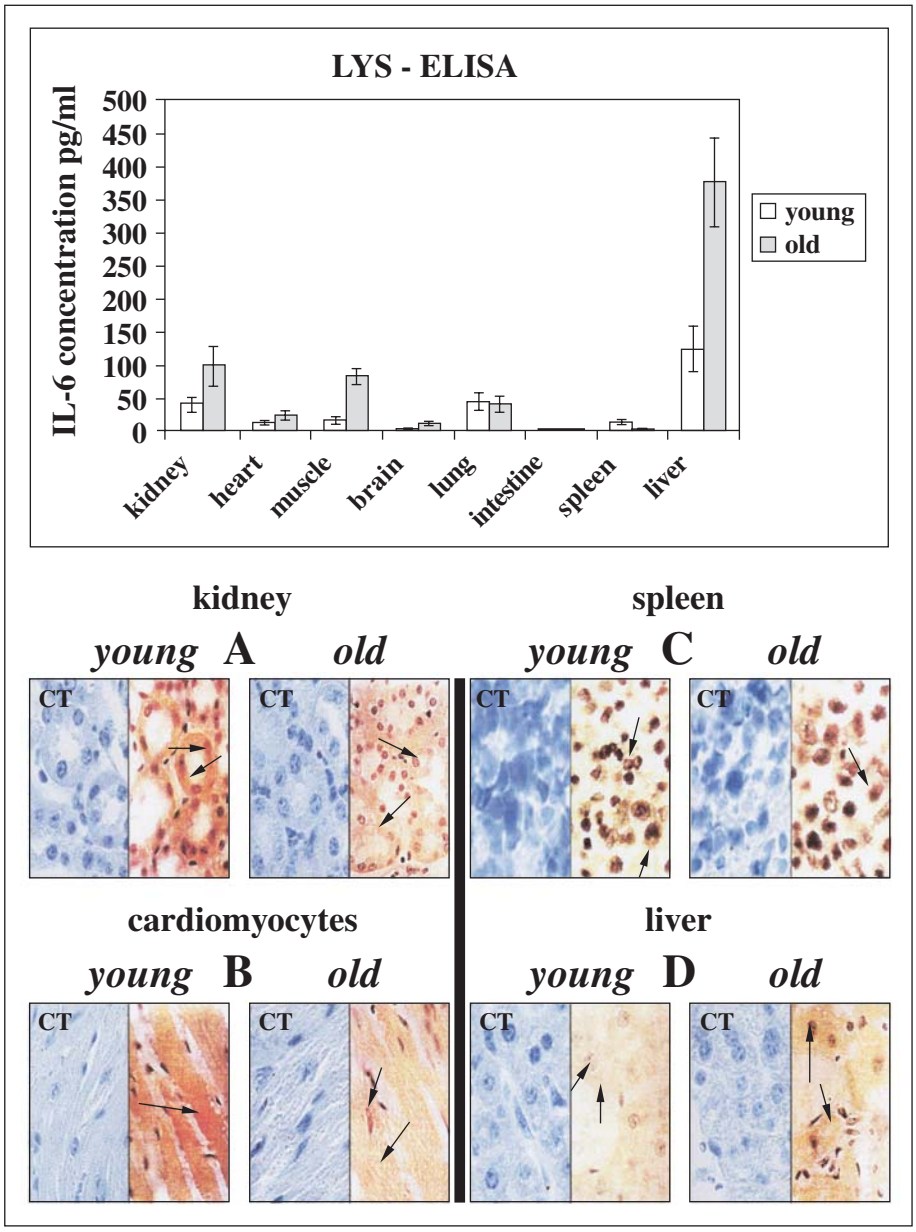

Figure 1

Intracellular organ expression of IL-6 in young versus old mice assessed by ELISA and immunohistochemistry. The presence of immunoreactive IL-6 in organ lysates was detected by commercial ELISA kits and is comparatively presented for young (empty bars) and old (solid bars) mice. IL-6 expression, manifested by the red-brown staining by the DAB dye, was characterized in the nuclei (upper arrows, A) and cytoplasm (lower arrows, A) of kidney tubular cells, cytoplasm (arrow in young mice, lower arrow in old mice, B) and nuclei (upper arrow, old mice, B) of cardiomyocytes, cytoplasm of spleen mononuclear cells (arrow, C), and nuclei (upper arrows, D), and cytoplasm (lower arrows, D) of liver hepatocytes of young and old mice by immunohistochemistry, using formalin-fixed, paraffin-embedded sections, as described in the Materials and Methods section. The specificity of IL-6 was determined using specific biotinylated rat anti-mouse monoclonal antibodies to IL-6. "CT" denotes control histological sections stained in the absence of the primary, specific antibody. Magnification of all figures is x 800 with the exception of the spleen (× 1600).

Similarly, a decreased expression was also shown for IL-10 in the heart *(Figure 2, B)*, skeletal muscle and brain (data not shown). In the kidney, IL-10 was demonstrated at comparable levels in both young and old mice *(Figure 2, A)*.

The expression of these cytokines was, in general, unaltered in the spleen and liver ((*Figure 1* for IL-6, *Figure 2* for IL-10), lungs and small intestine (data not shown), overall displaying a relative, low level of expression.

IL-6 and IL-10 expression in organ LYS is relatively low

In general (with the exception of the liver), IL-6 levels, as detected by ELISA in organ LYS under steady-state conditions, are low, hardly above the threshold of detection *(Figure 1)*. In young mice, levels of 41.3, 13.5, 16.1, 2.5 pg/ml were detected in the kidney, heart, skeletal muscle, and brain, respectively. In the lymphoreticular

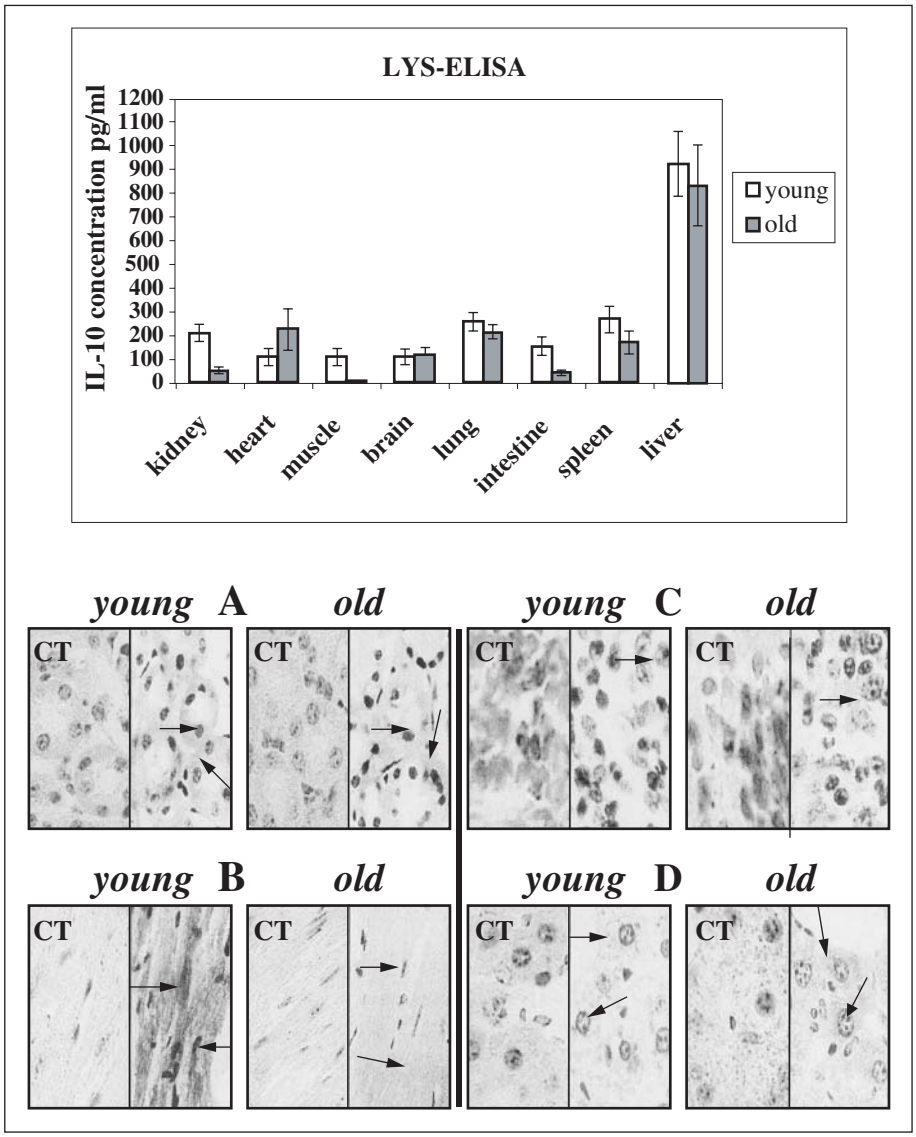

Figure 2

Intracellular organ expression of IL-10 in young versus old mice assessed by ELISA and immunohistochemistry. The presence of immunoreactive IL-10 in organ lysates was detected by commercial ELISA kits and is comparatively presented for young (empty bars) and old (solid bars) mice. IL-10 expression, manifested by the red-brown staining by the DAB dye, was characterized in the nuclei (upper arrows, A) and cytoplasm (lower arrows, A) of kidney tubular cells, cytoplasm (upper arrow in young mice, lower arrow in old mice, B) and nuclei (lower arrow in young mice, upper arrow in old mice, B) of cardiomyocytes, cytoplasm of spleen mononuclear cells (arrow, C), and nuclei (lower arrows, D, and cytoplasm (upper arrows, D) of liver hepatocytes of young and old mice by immunohistochemistry, using formalin-fixed, paraffin-embedded sections, as described in the Materials and Methods section. The specificity of IL-6 was determined using specific biotinylated rat anti-mouse monoclonal antibodies to IL-6. "CT" denotes control histological sections stained in the absence of the primary, specific antibody. Magnification of all figures is x 800 with the exception of the spleen (× 1600).

organs, IL-6 levels of 45.2, 2, 13.3 pg/ml were detected, respectively in the lungs, small intestine and spleen *(Figure 1)*. No significant difference was found in the two categories of organs (in general, $p > 0.05$ for most organ comparisons). In old mice, comparable low levels of IL-6 were detected in most organs ($p > 0.05$) with the exception of the kidney (97.97 pg/ml, $p < 0.05$ for most relative organ comparisons) and skeletal muscle (82.74 pg/ml, similar $p < 0.05$ values), in which significantly elevated IL-6 levels were detected *(Figure 1)*.

Nevertheless, even in this latter case, IL-6 did not exceed the level of 100 pg/ml. The only exception in this respect was noted in the liver, which displayed much higher levels of IL-6 *(Figure 1)*.

Similarly to IL-6, IL-10 levels in organ LYS, as detected by ELISA, were in general uniformly low (with the exception of the liver, *Figure 2*), as compared with the much higher levels disclosed in the CM obtained from the kidney, heart and liver *(Figure 3)*. Thus, in the internal, highly specialized organs of young mice, IL-10 values ranged from 108.1 pg/ml (heart and brain) to 209.3 pg/ml (kidney). The corresponding values in old mice were 224.8, 118.9 and 51.3 pg/ml, respectively (p values for the respective old/young organ differences were 0.023, 0.607 and < 0.001). Similar patterns of cytokine expression were displayed in the lymphoreticular organs: 259.3, 156.4 and 269.2 pg/ml in the lungs, small intestine and spleen, respectively (young age), compared to corresponding values of 215.1, 44.4, 173.6 pg/ml in old mice ($p = 0.09$, < 0.001 and 0.018, respectively, *Figure 2*).

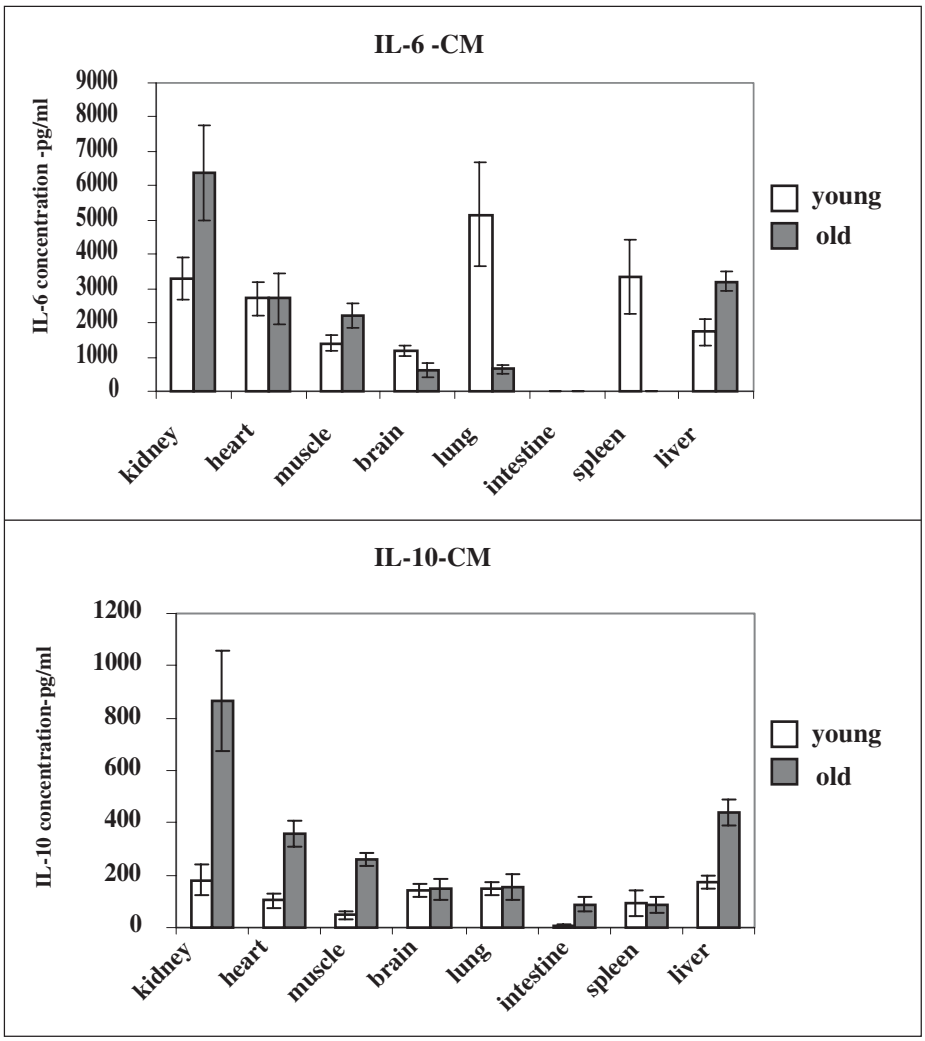

Figure 2

IL-6 versus IL-10 expression in organ CM. The expression of IL-6 and IL-10 in organ CM was assessed by ELISA in young (empty bars) and old mice (solid bars), as described in the Materials and Methods section, and is presented in pg/ml. The respective IL-6 and IL-10 expression for young and old mice is comparatively depicted for each organ. For better illustration, the sequence of organs shown was arranged based on the decreasing expression of IL-6 and IL-10 in each group of organs.

Increased relative expression of IL-6 and IL-10 in CM of highly specialized organs and liver in old mice

IL-6 and IL-10 were found at relatively enhanced levels in CM of the internal, specialized organs and liver of old mice, as assessed by ELISA *(Figure 3)*. Thus, in these mice, IL-6 was detected most markedly in the kidney, heart, skeletal muscle and liver (6361.86, 2703.23, 2224.58, 3204.83 pg/ml, respectively), compared with the lungs (652.83 pg/ml,), small intestine and spleen (non-detectable). The p value referring to the difference between the skeletal muscle, for example, and the lungs was 0.032 (statistically significant). In young mice, by comparison, the levels of IL-6 in the internal, specialized organs were comparable in magnitude (e.g. the kidney compared with the spleen, $p = 1.000$, *Figure 3)* or lower (e.g. the heart compared with the lungs, $p = 0.003$, *Figure 1)*, relative to the lymphoreticular organs. Similarly, the expression of IL-10 was significantly higher in the kidney, heart, skeletal muscle and liver (865.26, 357.9, 260.36 and 439.8 pg/ml, respectively), compared with the lungs, small intestine and spleen (153.76, 88.96 and 86 pg/ml, respectively, $p = 0.01$, for the difference between the skeletal muscle and the lungs, *Figure 3*). It should, however, be noted that aging leads to different cytokine-specific modulations in this respect: IL-6 appears to be dramatically down-regulated in the lungs and spleen, while IL-10 is markedly upregulated in kidney, heart, skeletal muscle and liver, relative to the generally lower level (up to ~ 200 pg/ml) found in young mice.

Also, of note is the almost an order of magnitude difference in the average value of organ IL-6 (2356.17 pg/ml for young mice and 1972.52 pg/ml for old mice) compared with IL-10 (111.5 pg/ml for young mice and 299.8 pg/ml for old mice), $p < 0.001$.

Relative IL-10 and IL-6 expression in organ CM versus organ LYS

In order to highlight possible differences in the cellular compartmentalization of these cytokines in the two age groups, the old/young ratio, with regard to IL-6 and IL-10 organ expression in young and old mice was addressed. The calculation of the ratio was based on the respective values relating to IL-6 and IL-10 expression, as detected in the young and old mice by the ELISA analyses. As shown in *Figure 4* for CM, relative expression of IL-10 consider-

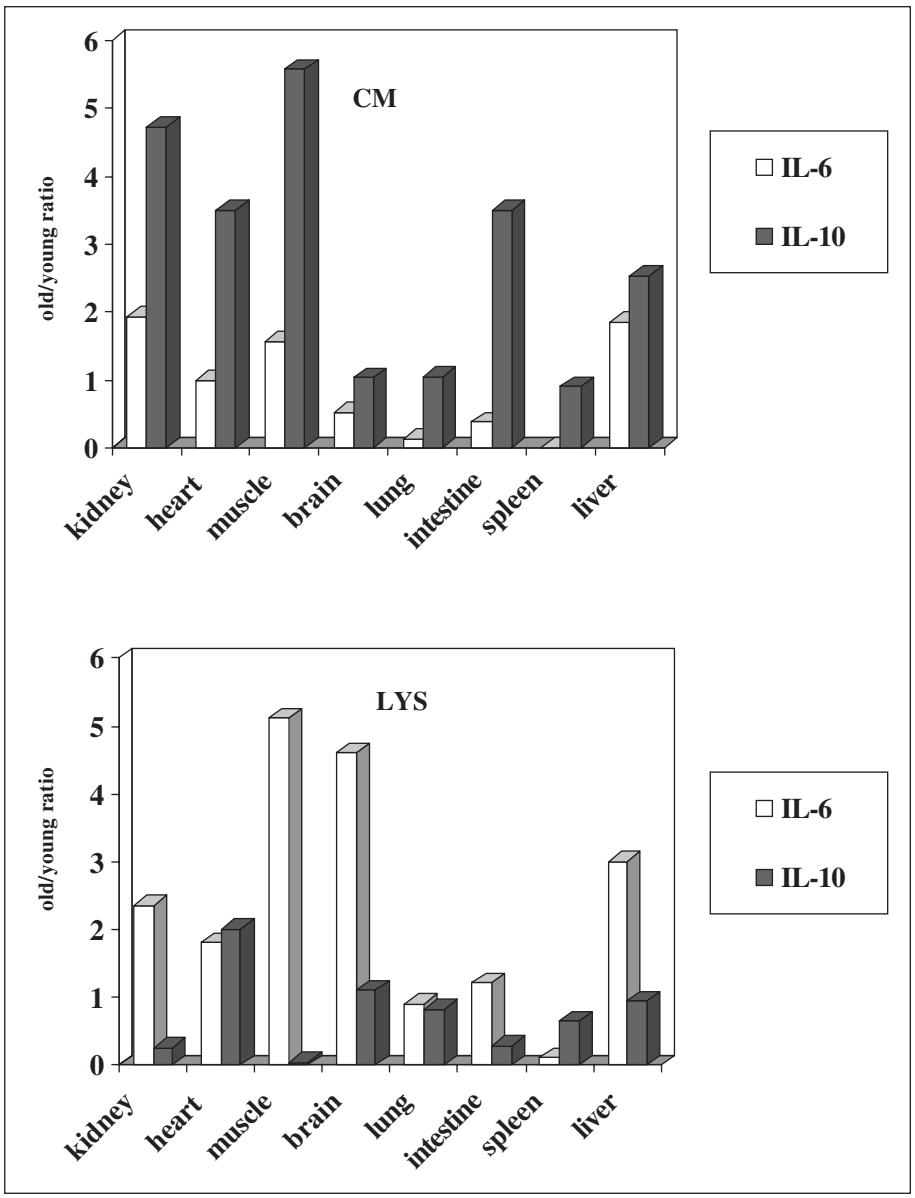

Figure 4

Relative organ expression of IL-6 and IL-10 in old versus young mice. The old/young ratio of organ cytokine expression was calculated, based on IL-6 and IL-10 values, as detected in young and old mice by ELISA. The respective values, obtained by ELISA for organ CM (A) and LYS (B) are comparatively presented for IL-6 (solid bars) and IL-10 (empty bars).

ably exceeds that of IL-6 with aging in most organs. An inverse trend is seen in analyses of organ LYS. In this case (with the exception of the spleen) *(Figure 4)*, the relative (compared with IL-6) expression of IL-10 is either of comparable magnitude (heart and lungs) or reduced (kidney, skeletal muscle, brain, small intestine and liver). On the other hand, aging accentuates the relative expression of IL-6 in kidney, skeletal muscle, brain and liver LYS *(Figure 4)*. These differences may denote unique roles for IL-6 and IL-10 in the intracellular compartment and outside the cell, respectively, in organ homeostasis and disease.

The results presented here refer to different physiological states and cytokine expression in the different compartments of organs. On one hand, intracellular cytokine expression in organs found at steady-state was analyzed by two methods (IHC and ELISA analyses of LYS), which, in principle, can provide complementary data. It should also be noted that by tissue homogenizing, quick, stress-induced alterations could, in principle, be observed in the intracellularly-expressed cytokines (not necessarily prevented by the use of protease inhibitors). On the other hand, detection of significant levels of IL-6 and IL-10 in organ CM imply active secretion or diffusion of the cytokine molecules from intracellular reservoirs (e.g. bound to cellular membranes) and/or cytokine generation upon the stressful conditions of organ culturing.

The apparent, non-correlating trends in IL-6 and IL-10 organ expression upon aging can thus be more easily understood. For example, reduced IHC staining in old animals may denote an absolute decrease in the intracellular cytokine level. Alternatively, post-translational modifications in the cytokine molecule, that might affect the binding of detecting antibodies, could also be envisioned (e.g. involving cytokine glycosylation, [41-43]). Evidently, this is not in contradiction with an increased

production/secretion of the cytokine into the environment upon stress (increased detection in organ CM).

DISCUSSION

The findings presented in this work point to specific patterns of IL-6 and IL-10 expression in organs, in young and old mice, as distinctly demonstrated by immunohistochemistry and ELISA of organ LYS and CM. This may help clarify the roles of IL-6 and IL-10 in homeostasis/defense under varying physiological/pathological conditions.

Based on the findings obtained by the cytoplasmatic IHC staining of fresh organs, IL-6 and IL-10 emerge as more dominantly expressed in the heart, skeletal muscle, brain and kidney (only for IL-6) of young mice, probably denoting a more influential, organ-protective role in these highly vulnerable organs. This is in line with previous evidence, showing a relative increased IL-6 bioactivity in CM obtained from these organs in young mice [24], despite the overall similar level of organ expression shown here by ELISA. These discrepant data emphasize the importance of inhibitors and modulators of IL-6 bioactivity, such as soluble IL-6R and gp130 [20, 21], and the local network of cytokine-cytokine receptors [44], when addressing the issue of organ IL-6 expression.

As a function of their anti-inflammatory and anti-apoptotic potential, the overall low levels of IL-6 and IL-10 detected in organ LYS may point to a basic tissue-preserving mechanism. Even the relatively high level of ELISA-detected IL-6 in the liver LYS, as here shown, may not necessarily reflect elevated biological activity. Indeed, we have observed low levels of IL-6 bioactivity in liver LYS in young mice [26]. In this context, the elevated levels of IL-6 *(Figure 1)* and downregulation of IL-10 *(Figure 2)* in LYS obtained from skeletal muscle and kidney of old mice may signify unique, cytokine-based functions.

The most prominent age-related alterations in cytokine expression were observed in organ CM: aging appears to result in a dramatic reduction of IL-6 levels in the lymphoreticular organs (with the exception of the liver), whereas IL-10 expression is markedly upregulated in the highly specialized organs (with the exception of the brain). It thus seems that under the conditions imposed by organ culturing in our studies, IL-6 secretion/production in old animals remains essential in the highly specialized organs (it is not downregulated, *Figure 4*). On the other hand, the potential of these organs to secrete/generate IL-10 in old age appears remarkably elevated (versus young age), suggesting a more pronounced impact of IL-10 on the homeostasis of highly specialized organs in aged animals.

In principle, both IL-6 and IL-10 could play an important, tissue-preserving role in highly vulnerable organs, in view of their inflammation and apoptosis-restraining roles. However, the relative increased IL-10 expression (versus IL-6) in this context may denote a regulatory and perhaps complementary effect vis-a-vis IL-6.

Aging cells are more sensitive to stressful (apoptosis-inducing) stimuli and their capability to recuperate after such events is reduced [45, 46]. In this context, survival of aging neurons under stressing conditions appears much more dependent on trophic, gp130-activating cytokines [47], suggesting an important role for IL-6 in organ homeostasis under these conditions. On the other hand, IL-6 seems to be generally involved in various destructive processes of aging [48]; IL-6 can mediate an increased thrombotic state [16], a negative inotropic effect on myocardial tissue [49], cardiac hypertrophy [50], muscle atrophy [51], growth retardation [52], progression of autoimmune and neoplastic processes [53], all processes typical of advancing age. Some of these noxious IL-6-mediated, effects could derive from age-related alterations in "beneficial" transducing pathways downstream gp130 activation, such as described for DNA-repair activity mediated by STAT [54]. In this respect, IL-10 might contribute to tightly regulating the expression of organ IL-6 and restraining inflammation/apoptosis in highly specialized and vulnerable organs of old animals, especially under stressful conditions. Indeed, IL-10 can directly downregulate IL-6 expression [55, 56], as also suggested by the linked decline in IL-10 and increased expression of IL-6 in brains of old mice [57]. Also, IL-10-mediated transduction through STAT may preferentially (over IL-6) be activated in this setting (e.g., potentially through the gp130-modulating suppressor of cytokine signalling 3 -SOCS3-, 58), alteration in STAT-mediated transduction upon aging has indeed been described [59]. IL-10 protection of brain [60] and heart [34], its involvement in FasL-induced immune privilege status [61] and the stimulation of various cell populations involved in immunesurveillance [62], further attest to its possible organ-preserving role. When dysregulated however, this seemingly adapting patterns of IL-6 and IL-10 organ expression may perturb the balanced effects of the immune system in aged animals.

In the lymphoreticular organs, by contrast, the reduced/altered expression of IL-6 and IL-10 may enable adjustment of adequate levels of tissue protection, and which appears to be based on powerful proinflammatory cytokines, such as IL-1 [26, 63]

The expression of IL-6 and IL-10 in the liver, overall increases considerably in old mice (unlike the other lymphoreticular organs tested). The liver's distinct detoxifying functions and its unique role in body homeostasis and defense (generating and vitally regulating critical factors such as blood glucose, albumin and the acute phase proteins), probably explain this behavior. Indeed, IL-6 [17-19] and IL-10 [64] emerge as liver-protective.

Overall, organ-specific expression of IL-6 and IL-10 and modulation by aging, as shown here, strongly suggests the presence of complex cytokine-based networks, specifically adapting to the unique functional needs and characteristics of the organ. A thorough understanding of such networks may hopefully enable us to counteract or reduce age-related deterioration in body defense.

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REFERENCES

1. Hirano T. 1998. Interleukin-6 and its receptor: ten years later. *Int. Rev. Immunol.* 16: 249.
2. Barton BE. 1997. IL-6: Insights into novel biological activities. *Clin. Immunol. Immunopathol.* 85: 15.
3. Akira S, Taga T, Kishimoto T. 1993. Interleukin-6 in biology and medicine. *Adv. Immunol.* 54: 1.
4. Baumann H, Gauldie J. 1994. The acute phase response. *Immunol. Today* 15: 74.
5. Tilg H, Dinarello CA, Mier JW. 1997. IL-6 and APPs: anti-inflammatory and immunosuppressive mediators. *Immunol. Today* 18: 428.
6. Seiler P, Plenz G, Deng MC. 2001. The interleukin-6 cytokine system in embryonic development, embryo-maternal interactions and cardiogenesis. *Eur. Cytokine Netw.* 12: 15.
7. Chen L, Mory Y, Zilberstein A, Revel M. 1988. Growth inhibition of human breast carcinoma and leukemia/lymphoma cell lines by recombinant interferon beta 2. *Proc. Natl. Acad. Sci. U.S.A* 85: 8037.
8. Lu C, Kerbel O. 1993. Interleukin-6 undergoes transition from paracrine growth inhibitor to autocrine stimulator during human melanoma progression. *J. Cell. Biol.* 120: 1281.
9. Bihl M, Tamm M, Nauck M, Wieland H, Perruchoud AP, Roth M. 1998. Proliferation of human non-small-cell lung cancer cell lines: role of interleukin-6. *Am. J. Respir. Cell. Mol. Biol.* 19: 606.
10. Moreno A, Villar ML, Camera C, Luque R, Cespon C, Gonzales-Porque P, Roy G, Lopez-Jimenez J, Bootello A, Santiago ER. 2001. Interleukin-6 dimers produced by endothelial cells inhibit apoptosis of B-chronic lymphocytic leukemia cells. *Blood* 97: 242.
11. Chung TD, Yu JJ, Kong TA, Spiotto MT, Lin JM. 2000. Interleukin-6 activates phospatidylinositol-3 kinase, which inhibits apoptosis in human prostate cancer cell lines. *Prostate* 42: 1.
12. Mitsiades CS, Mitsiades N, Poulaki V, Schlossman R, Akiyama M, Chauhan D, Hideshima T, Treon SP, Munshi NC, Richardson PG, Anderson KC. 2002. Activation of NF-kappaB and upregulation of intracellular anti-apoptotic proteins via the IGF-1/Akt signaling in human multiple myeloma cells: therapeutic implications. *Oncogene* 21: 5673.
13. Narimatsu M, Maeda H, Itoh S, Atsumi T, Ohtani T, Nishida K, Itoh M, Kamimura D, Park SJ, Mizuno K, Miyazaki J, Hibi M, Ishihara K, Nakajima K, Hirano T. 2001. Tissue-specific autoregulation of the stat3 gene and its role in interleukin-6-induced survival signals in T cells. *Mol. Cell. Biol.* 21: 6615.
14. Hong F, Nguyen VA, Shen X, Kunos G, Gao B. 2000. Rapid activation of protein kinase B/Akt has a role in antiapoptotic signaling during liver regeneration. *Biochem. Biophys. Res. Commun.* 279: 974.
15. Maeda Y, Matsumoto M, Hori O, Kuwabara K, Ogawa S, Yan SD, Ohtsuki T, Kinoshita T, Kamada T, Stern DM. 1994. Hypoxia/reoxygenation-mediated induction of astrocyte IL-6: a paracrine mechanism potentially enhancing neuron survival. *J. Exp. Med.* 180: 2297.
16. Kerr R, Stirling D, Ludlam CA. 2001. Interleukin-6 and haemostasis. *Br. J. Haematol.* 115: 3.
17. Hong F, Kim WH, Tian Z, Jaruga B, Ishac E, Shen X, Gao B. 2002. Elevated interleukin-6 during ethanol consumption acts as a potential endogenous protective cytokine against ethanol-induced apoptosis in the liver: involvement of induction of Bcl-2 and Bcl-x(L) proteins. *Oncogene* 21: 32.
18. Streetz KL, Luedde T, Manns MP, Trautwein C. 2000. Interleukin-6 and liver regeneration. *Gut* 47: 309.
19. Ezure T, Sakamoto T, Tsuji H, Lunz JG. 3rd, Murase N, Fung JJ, Demetris AJ 2000. The development and compensation of biliary cirhosis in interleukin-6 deficient mice. *Am. J. Pathol.* 156: 1627.
20. Kamimura D, Ishihara K, Hirano T. 2003. IL-6 signal transduction and its physiological roles: the signal orchestration model. *Rev. Physiol. Biochem. Pharmacol.* 149: 1.
21. Nowell MA, Richards PJ, Horiuchi S, Yamamoto N, Rose-John S, Tople N, Williams AS, Jones SA. 2003. Soluble IL-6 receptor governs IL-6 activity in experimental arthritis: blockade of arthritis severity by soluble glycoprotein 130. *J. Immunol.* 171: 3202.
22. Moore KW, Malefyt RW, Coffman RL, O'Garra A. 2001 Interleukin-10 and the interleukin-10 receptor. *Ann. Rev. Immunol.* 19: 683.
23. Deng J, Kohda Y, Chiao H, Wang Y, Hu X, Hewitt SM, Miyaji T, MeLeroy P, Nibhanupudy B, Li S, Star RA. 2001. Interleukin-10 inhibits ischemic and cisplatin-induced acute renal injury. *Kidney* Int 60: 2118.
24. Oberholzer C, Oberholzer A, Bahjat FR, Minter RM, Tannahill CL, Abouhamze A, LaFace D, Hutchins B, Clare-Salzer MJ, Moldawer LL. 2001. Targeted adenovirus-induced expression of IL-10 decreases thymic apoptosis and improves survival in murine sepsis. *Proc. Natl. Acad. Sci. USA* 98: 11503.
25. Zhou JH, Broussard SR, Strle K, Freund GG, Johnson RW, Dantzer R, Kelley KW. 2001. IL-10 inhibits apoptosis of promyeloid cells by activating insulin receptor substrate-2 and phosphatidylinositol 3'-kinase. *J. Immunol.* 167: 4436.
26. Hacham M, Cristal N, White RM, Segal S, Apte RN. 1996. Complementary organ expression of IL-1 versus IL-6 and CSF-1 in normal and LPS-injected mice. *Cytokine* 8: 21.
27. Gwechenberger M, Mendoza LH, Youker KA, Frangogiannis NG, Smith CW, Michael LH, Entman ML. 1999. Cardiac myocytes produce interleukin-6 in culture and in viable border zone of reperfused infarctions. *Circulation* 99: 546.
28. Saito H, Patterson C, Hu Z, Runge MS, Tipnis U, Sinha M, Papaconstantinou J. 2000. Expression and self-regulatory function of cardiac interleukin-6 during endotoxemia. *Am. J. Physiol. Heart. Circ. Physiol.* 279: H2241.
29. Pederson BK, Steensberg A, Schjerling P. 2001 Muscle-derived interleukin-6: possible biological effects. *J. Physiol.* 536: 329.
30. Brosk AP, Halloran PF. 1994. Tissue distribution of IL-10 mRNA in normal mice. Evidence that a component of IL-10 expression is T and B cell-independent and increased by irradiation. *Transplantation* 57: 582.
31. Van Cott JL, Staats HF, Pascual Dwet *et al.* 1996. Regulation of mucosal and systemic antibody responses by T helper cell subsets, macrophages and derived cytokines following oral immunization with live recombinant Salmonella. *J. Immnol.*. 156: 1504.
32. Berg DJ, Davidson N, Kuhn R *et al.* 1996. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J. Clin. Invest.* 98: 1010.
33. Sturlan S, Oberhuber G, Beinhauer BG, Tichy B, Kappel S, Wang J, Rogy MA. 2001. Interleukin-10-deficient mice and inflammatory bowel disease associated cancer development. *Carcinogenesis* 22: 665.
34. Jones SP, Trocha SD, Lefer DJ. 2001. Cardioprotective actions of endogenous IL-10 are independent of iNOS. *Am J Physiol Heart Circ Physiol* 281: H48.
35. Sharman K, Sharman E, Campbell A, Bondy SC. 2001. Reduced basal levels and enhanced LPS response of IL-6 mRNA in aged mice. *J. Gerontol. Biol. Sci.* 12: B 520.
36. Miller R. 1996. The aging immune system: Primer and Prospectus. *Science* 273: 70.
37. Doria G, Frasca D. 1997. Genes, immunity, and senescence: looking for a link. *Immunol. Rev.* 160: 159.
38. Gardner EM, Murasko DM. 2002. Age-related changes in Type 1 and Type 2 cytokine production in humans. *Biogerontology* 3: 271.

39. Maisy K, Nardocci G, Imarai M, Cardenas H, Rios M, Croxatto HB, Heckels JE. 2003. Expression of proinflammatory cytokines and receptors by human falopian tubes organ culture following challenge with Neisseria gonorrhea. *Infect. Immun.* 71: 527-532.

40. Bellone G, Turletti A, Artusio E, Mareschi K, Carbone A, Tibaudi D, Robecchi A, Emanuelli G, Rodeck U. 1999. Tumor-associated transforming factor-beta and interleukin-10 contribute to a systemic Th2 immune phenotype in pancreatic carcinoma patients. *Am. J. Pathol.* 155: 537.

41. Parekh RB, Dwek RA, Rademacher TW, Opdenakker G, Van Damme J. 1992. Glycosylation of interleukin-6 purified from normal human blood mononuclear cells. Eur. J. *Biochem.* 203: 135.

42. May LT, Shaw JE, Khanna AK, Zabriskie JB, Sehgal PB. 1991. Marked cell-type-specific differences in glycosylation of human interleukin-6. *Cytokine* 3: 204.

43. Reineke V, Schneider-Mergener J, Glasser RW, Stigler RD, Seifert M, Volk HD, Sabat R. 1999. Evidence for conformationally different states of interleukin-10: binding of a neutralizing antibody enhances accessibility of a hidden epitope. *J. Mol. Recognit.* 12: 242.

44. Ahmed ST, Ivashkiv LB. 2000. Inhibition of IL-6 and IL-10 signalling and Stat activation by inflammatory and stress pathways. *J. Immunol.* 165: 5227.

45. Mariani J, Ou R, Bailey M, Rowland M, Nagley P, Rosenfeldt F, Pepe S. 2000. Tolerance to ischemia and hypoxia is reduced in aged human myocardium. *J. Thorac. Cardiovasc. Surg.* 120: 660.

46. Ikeyama S, Wang XT, Li J, Podlutsky A, Martindale JL, Kokkonen, Huizen R, Gorospe M, Holbrook NJ. 2003. Expression of the pro-apoptotic gene gadd153/chop is elevated in liver with aging and sensitizes cells to oxidant injury. *J. Biol. Chem.* 278: 16726.

47. Schweizer U, Gunnersen J, Karch C, Wiese S, Holtmann B, Takeda K, Akira S, Sendtner M. 2002. Conditional gene ablation of Stat3 reveals differential signaling requirements for survival of motorneurons during development and after nerve injury in the adult. *J. Cell. Biol.* 156: 287.

48. Ershler WB, Keller ET. 2000. Age-associated increased interleukin-6 gene expression, late-life diseases and frailty. *Annu Rev Med* 51: 245.

49. Villegas S, Villarreal FJ, Dillmann WH. 2000. Leukemia inhibitory factor and interleukin-6 downregulate sarcoplasmic reticulum Ca2 + ATPase (SERCA2) in cardiac myocytes. *Basic. Res. Cardiol.* 95: 47.

50. Sano M, Fukuda K, Kodama H, Pan J, Saito M, Matsuzaki J, Takashi T, Makino S, Kato T, Ogawa S. 2000. Interleukin-6 family of cytokines mediate angiotensin II- induced cardiac hypertrophy in rodent cardiac myocytes. *J. Biol. Chem.* 275: 29717.

51. Tsujinaka T, Fujita J, Ebisui C, Yano M, Kuminami E, Suzuki K, Tanaka K, Katsume A, Ohsugi Y, Shiozaki H, Monden M. 1996. Interleukin-6 receptor antibody inihibits muscle atrophy and modulates proteolytic systems in interleukin-6 transgenic mice. *J. Clin. Invest.* 97: 244.

52. Lieskovska J, Guo D, Derman E. 2002. IL-6-overexpression brings about growth impairement potentially through a GH receptor deficit. *Growth Horm IGF Res.* 12: 388.

53. Ishikara K, Hirano T. 2002. IL-6 in autommune disease and chronic inflammatory proliferative disease. *Cytokine Growth Factor Rev.* 13: 357.

54. Frasca D, Scarpaci S, Barattini P, Bartoloni C, Guidi L,Costanzo M, Doria G. 2002. The DNA repair protein ku is involved in gp130-mediated signal transduction events in PBMC from young but not from elder subjects. *Exp. Gerontol.* 37: 321.

55. de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. 1991. Interleukin-10 (IL-10) inhibits cytokine synthesis by human monocytes: An autoregulatory role of IL-10 produced by monocytes. *J. Exp. Med.* 174: 1209.

56. Heyen JRR, Ye SM, Finck BN, Johnson RW. 2000. Interleukin (IL)-10 inihibits IL-6 production in microglia by preventing activation of NF-RB. *Mol. Brain. Res.* 77: 138.

57. Shi-Ming Ye, Johnson RW. 2001. An age-related decline in interleukin-10 may contribute to the increased expression of interleukin-6 in brain of aged mice. *Neuroimmunomodulation* 9: 183.

58. Lang R, Pauleau AL, Parganas E, Takahashi Y, Mages J, Ihle JN, Rutschman R, Murray PJ. 2003. SOCS3 regulates the plasticity of gp130 signalling. *Nat. Immunol.* 4: 546-550.

59. De-Fraja C, Conti L, Govoni S, Battaini F, Cattaneo E. 2000. STAT signalling in the mature and aging brain. *Int. J. Dev. Neurosci.* 18: 439.

60. Strle K, Zhou JH, Shen WH, Broussard SR, Johnson RW, Freund GG, Dantzer R, Kelly KW. 2001. Interleukin-10 in the brain. *Crit. Rev. Immunol.* 21: 427.

61. Tourneur L, Damotte D, Marion S, Mistou S, Chiocchia G. 2002. IL-10 is necessary for FasL-induced protection from experimental autoimmune thyroiditis but not for FasL-induced immune deviation. *Eur. J. Immunol.* 32: 1292.

62. Morcellin S, Panelli MC, Wang E, Nagorsen D, Marincola FM. 2003. The dual role of IL-10. *Trends Immunol.* 24: 36-43.

63. Hacham M, Argov S, White RM, Segal S, Apte RN. 2002. Different patterns of interleukin-1α and interleukin-1β expression in organs of normal young and old mice. Eur *Cytokine Netw.* 13: 55.

64. Kato M, Ikeda N, Matsushita E, Kaneko S, Kobayashi K. 2001. Involvement of IL-10, an anti-inflammatory cytokine, in murine liver injury induced by Concavalin A. *Hepatol. Res.* 20: 232.