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Short report

Age-related dynamics of constitutive cytokine transcription levels of feline monocytes

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Abstract

Monocytes/macrophages are central mediators of inflammation and immunity and therefore of major interest in the study of immunosenescence. In healthy adult cats, monocytes have been shown to constitutively transcribe pro- and anti-inflammatory cytokines. However, in order to characterize the effect of age, feline monocyte functions were examined for changes in cytokine transcription levels in early stages of immunosenescence. For this purpose, isolated, short-term cultured monocytes from barrier-maintained adult cats of different ages (15 mo to 10 yr) were examined for transcription of IL-1β, IL-6, IL-10, IL-12 p40 and TNF-α by real-time PCR. Transcription levels of cytokines varied and were generally highest for IL-1β. For IL-1β, IL-6 and IL-12 p40, both young and old cats exhibited highest levels. The age association was significant. TNF-α appeared to be transcribed at similar levels over the examination period, whereas IL-10 tended to decline with age but without any statistical significant differences. The observed age association of the constitutive transcription of some cytokines indicates a drop in monocyte activities from youth to middle age, which is then followed by a (progressive) increase with increasing age. This provides evidence that monocytes are in part responsible for the pro-inflammatory status observed with ageing.
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1. Introduction

Monocytes/macrophages play the central role in inflammatory responses, immunity as well as the stress response (Franceschi et al., 2000). Furthermore, a chronic, age-related, progressive stimulation of macrophages towards a pro-inflammatory status was postulated and known as 'macroph-aging' (Franceschi et al., 2000). One previous study actually showed significantly increased constitutive production of IL-1β and IL-6 in circulating monocytes of older female compared to young adult female human individuals, whereas no changes in the constitutive

TNF-α production were observed (Sadeghi et al., 1999). On the other hand, there is evidence for decreased in vitro production of some cytokines, such as IL-1β, IL-6 and TNF-α, in LPS-stimulated whole blood or monocytes of elderly humans (Bruunsgaard et al., 1999; Sadeghi et al., 1999). In aged mice, a reduced capacity was observed of activated macrophages to produce IL-1β, TNF-α and nitric oxide (Wallace et al., 1995). Furthermore, aged rat monocytes/macrophages exhibit a reduced constitutive MCP-1 production (Reale et al., 2003).

There is a paucity of information on the constitutive production of cytokines by monocytes/macrophages, for all species. A previous study on human monocytes demonstrated constitutive production of IL-1β, IL-6 and TNF-α by unstimulated human monocytes in young and old females (Sadeghi et al., 1999); whereas another study demonstrated variable constitutive transcription of IL-1β, IL-6, IL-10, IL-12p40 and TNF-α in isolated, short term-cultured,

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unstimulated monocytes in cats of different ages (Kipar et al., 2001). However, with regard to monocyte-associated viral infections in the cat, certain age-related differences in susceptibility have been noted. For example, feline infectious peritonitis (FIP), a coronavirus-induced, immune-mediated, fatal systemic disease, affects most frequently young animals between 6 mo and 2 yr of age (Rohrbach et al., 2001). However, aged cats develop a less intense immune response, with delayed antibody production to experimental feline immunodeficiency virus (FIV) infection compared to young animals (George et al., 1993). In both infectious diseases, the infected monocyte is essential for viral distribution (Magnani et al., 1994; Kipar et al., 2004, 2005) and in cases of FIP, the activated monocyte also mediates the development of its pathogenetic hallmark, granulomatous phlebitis (Kipar et al., 2005). The question arises as to whether age-related constitutive changes in monocyte functions are responsible for potential differences in responses to infectious agents. Thus, the purpose of this study is to evaluate constitutive transcription levels of cytokines in cats over a large age range, from 15 mo to 10 yr of age.

2. Materials and methods

2.1. Animals

The study was performed on 17 healthy, female or male neutered (in equal proportions) uninfected barrier-maintained cats, kept under comparable conditions at the Veterinary Faculties in Zurich, Switzerland and Giessen, Germany. Four cats were sampled near 14 mo (13–15 mo) of age, two near 20 mo (20 and 21 mo), two near 26 mo (25–29 mo), two near 3 yr (2 yr 11 mo–3 yr 1 mo), two near 3 yr 10 mo (3 yr 9 mo–3 yr 11 mo) and five near 5 yr 1 mo (5 yr–5 yr 2 mo) during the examination period. Three cats were re-sampled at an older age: one cat at 8 yr and 8 mo (previously examined at 3 yr 10 mo), two at 10 yr (previously examined at 5 yr 1 mo).

2.2. Monocyte isolation, RNA extraction, cDNA production and real time PCR for feline (f) IL-1β, fIL-6, fIL-10, fIL-12 p40 and fTNF-α

Blood samples were generally taken twice from each animal, 3 weeks to 2 mo apart. Peripheral blood leukocytes were isolated and short-term cultured for 15–18 h. Monocytes were purified by thorough washing of plates to eliminate non-adherent cells, as previously described (Kipar et al., 2001). RNA was extracted from monocytes using a commercially available kit (RNeasy mini kit; QIAGEN, Hilden, Germany). Contaminating genomic DNA in RNA extracts was digested and cDNA synthesized according to published protocols (Kipar et al., 2001). Real-time TaqMan PCR, using an automated fluorometer (ABI Prism 7700 Sequence Detection System, Applied Biosystems, Weiterstadt, Germany) was used to quantify the relative levels of feline (f) IL-1β, fIL-6, fIL-10, fIL-12 p40 and fTNF-α transcription (Kipar et al., 2001). From each cDNA sample, parallel reactions were performed, in duplicates, in separate tubes for the detection of fGAPDH (internal control/calibrator), fIL-1β, fIL-6, fIL-10, fIL-12 p40 and fTNF-α. Amplification conditions, assay compositions, primer and probe concentrations were as previously described (Kipar et al., 2001).

2.3. Quantification of cytokine transcription

Relative quantitation of cytokine signals was done by the comparative C_T method and is reported as relative transcription or the n-fold differences, relative to the calibrator cDNA (fGAPDH). For each sample, differences between the target and internal control C_T were calculated and served to normalize for differences in the quantity of total nucleic acid added to each reaction and in the efficiency of the RT step. The C_T of the calibrator, fGAPDH, was subtracted from the C_T of each experimental sample to give the value ΔC_T. For samples where a cytokine mRNA signal was not observed after 45 cycles, a plausible ΔC_T was created. This value was ascertained from the lowest C_T for fGAPDH (obtained from the sample with the highest expression of GAPDH) subtracted from 45 (number of cycles). The resulting value was rounded to the next higher integer value. The amount of target was calculated by $2^{-\Delta C_T}$, resulting in evaluation of the experimental samples as an n-fold difference relative to the calibrator fGAPDH (Kipar et al., 2001).

2.4. Statistical analysis

Statistical analysis was performed, using the programme SAS version 8.2 computer software PROC MIXED (SAS Institute, Cary, NC, USA) A regression analysis was performed of the cytokine transcription levels versus age, both as a linear and quadratic function. A multivariable regression model was explored for all cytokines against age. Final models were assessed by looking at the parameter estimates, the model deviance and the distribution of the residual values.

For the identification of potential linear associations between transcription of the different cytokines and age, the Pearson correlation coefficients matrix was used.

3. Results

All cytokines were constitutively transcribed. IL-1β and TNF-α were detected in all samples. IL-6 was not detected in two samples (each one from a cat aged 3 yr 1 mo and 3 yr 11 mo), IL-10 was not detected in two samples (each one from a cat aged 3 yr 9 mo and 5 yr 2 mo), and IL-12p40 was not detected in four samples (each one from a cat aged 3 yr 1 mo and 3 yr 11 mo and two cats aged 5 yr 2 mo).
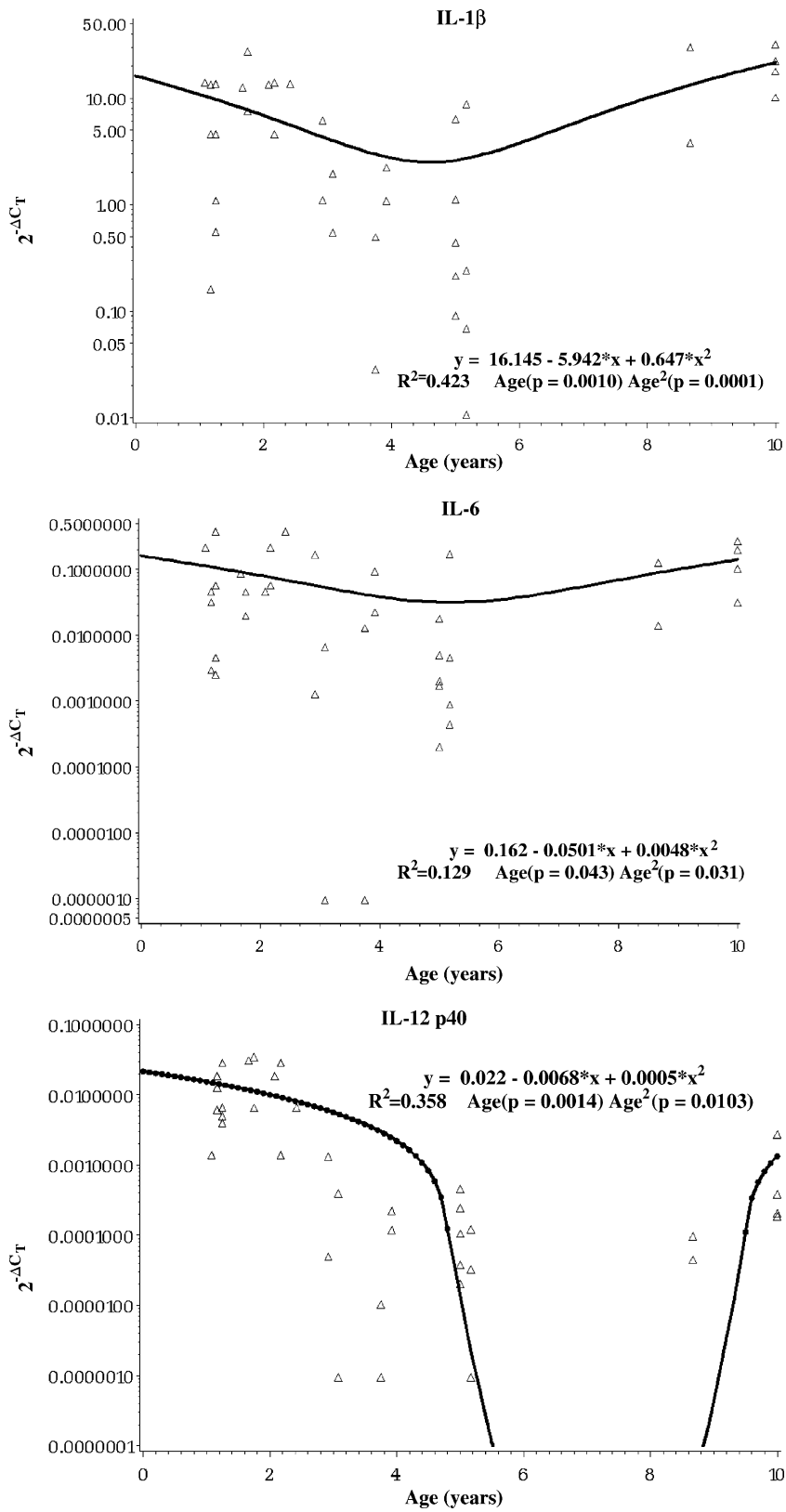
IL-1β

$$y = 16.145 - 5.942*x + 0.647*x^2$$
$$R^2 = 0.423 \quad Age(p = 0.0010) \; Age^2(p = 0.0001)$$

IL-6

$$y = 0.162 - 0.0501*x + 0.0048*x^2$$
$$R^2 = 0.129 \quad Age(p = 0.043) \; Age^2(p = 0.031)$$

IL-12 p40

$$y = 0.022 - 0.0068*x + 0.0005*x^2$$
$$R^2 = 0.358 \quad Age(p = 0.0014) \; Age^2(p = 0.0103)$$

Fig. 1. Associations of relative cytokine transcription levels (e.g. $2^{-\Delta C_T}$) with age. The value of $2^{-\Delta C_T}$, a standard method of expressing cytokine transcription levels, was calculated from the difference between the target and internal control C_T, as an *n*-fold difference relative to the calibrator fGAPDH. (Δ-individual cat cytokine value; •—• regression line from analysis).

IL-10

$$y = 0.256 - 0.080*x + 0.0055*x^2$$
$$R^2=0.049 \quad Age(p = 0.188) \quad Age^2(p = 0.199)$$

TNF-α

$$y = 0.081 - 0.0220*x + 0.0022*x^2$$
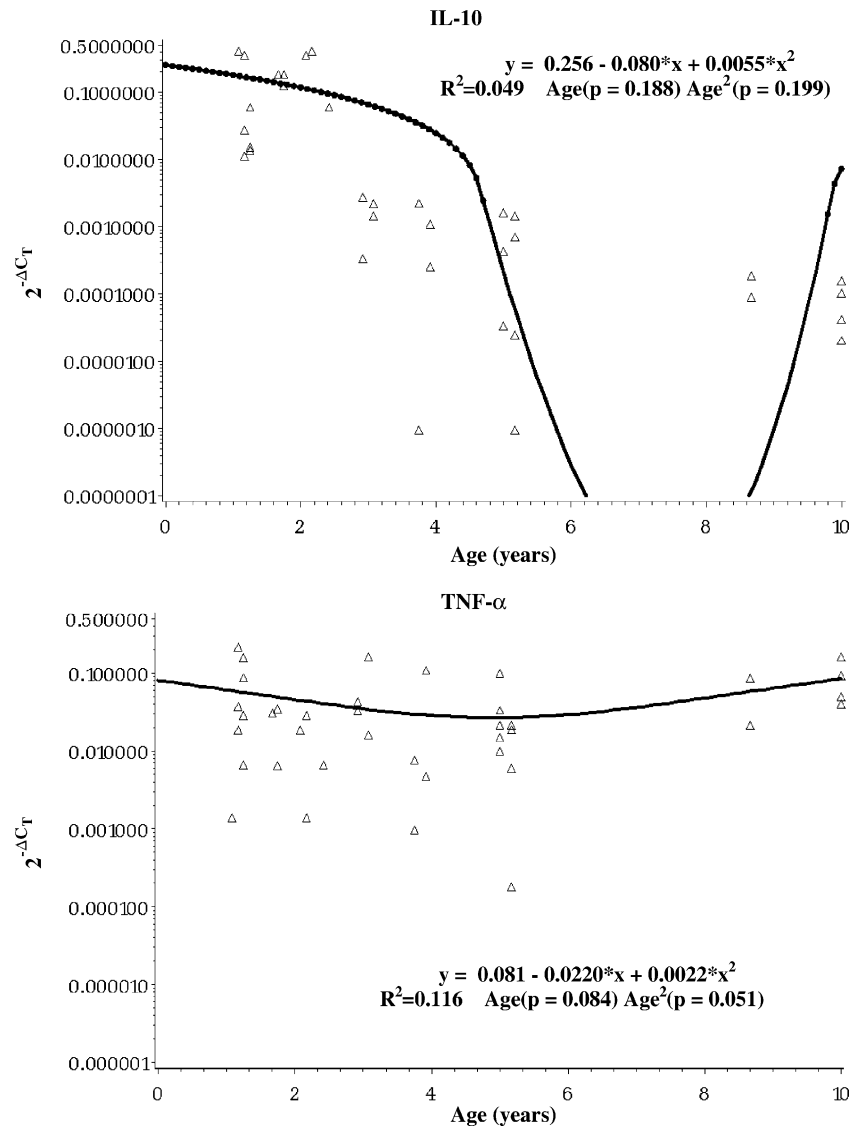$$R^2=0.116 \quad Age(p = 0.084) \quad Age^2(p = 0.051)$$

Fig. 1 (*continued*)

Transcription levels were generally variable, both among cats of the same age (Fig. 1) and in individual cats at different time points. IL-1β was overall transcribed most intensely, followed by TNF-α, IL-6 and IL-10. IL-12 p40 levels were generally very low (Fig. 1). For IL-1β, IL-6 and IL-12 p40, a statistically significant association was found between the level of transcription and age. Higher transcription levels were noted in younger and older cats than in those of middle age (Fig. 1). For IL-1β and IL-6, transcription levels were similar in the youngest and oldest cats, whereas IL-12 p40 transcription was highest in the youngest cats. For IL-10, an overall decline in constitutive transcription with age was indicated, but not statistically significant (Fig. 1). TNF-α levels were on average less variable throughout the examination period. A slight drop in transcription was observed in the middle-aged cats, but not statistically significant (Fig. 1). The multivariable regression analysis did not identify more suitable models

to evaluate the association between cytokine transcription levels and age. Results of the Pearson correlation coefficients matrix are illustrated in Table 1. Transcription of IL-1β and IL-6 appeared highly correlated, independent of age. A trend for a positive correlation between IL-1β and age was found as well as a moderate negative correlation between IL-12 p40 transcription and age.

Table 1
Pearson correlation coefficients matrix of all measured variables used in the analysis

	Age	IL-1β	IL-6	IL-19	IL-12 p40
IL-1β	0.331				
IL-6	0.052	0.598			
IL-10	0.050	−0.146	−0.127		
IL-12 p40	−0.472	0.262	−0.017	−0.085	
TNFα	0.114	0.128	−0.048	−0.113	−0.028

4. Discussion

This report presents the results of an initial study to assess the functional state of monocytes over a wide age range in cats, by evaluating a set of both pro-inflammatory and down-regulatory cytokines (e.g. IL-1β, IL-6, IL-10, IL-12 p40 and TNF-α), in isolated, short-time cultured, unstimulated monocytes. The age range of 15 mo to 10 yr in cats used in this study covers most of adult, pre-senile life of healthy cats. This study provides evidence for a decline in constitutive transcription of some cytokines (IL-1β, IL-6 and IL-12 p40) from early adulthood to middle age (approx. 5 yr), with their subsequent increase in older cats. The latter levels approach those seen in the youngest cats (for IL-1β and IL-6) or are comparatively lower (for IL-12 p40). IL-10 and TNF-α do not show any significant age-related changes in their constitutive transcription. Nonetheless, there was a tendency for a progressive decline in IL-10 transcription with age. TNF-α transcription levels, however, appear mostly stable over the entire age range, with a slight depression in the middle-aged cats.

Our study has two outcomes, namely to clearly emphasise the general importance of age-matched controls in studies on alterations of immune cell populations. Also, this study supports the hypothesis of a general, progressively increasing 'pro-inflammatory status' of monocytes/macrophages with age (Franceschi et al., 2000). It provides evidence that monocytes are at least partly responsible for the progressive increase in blood IL-6 and IL-1β levels observed in older individuals (Franceschi et al., 1999; Bruunsgaard et al., 1999). However, similar to a previous study on human monocytes (Sadeghi et al., 1999), the results of this study do not suggest involvement of monocytes in the induction of elevated TNF-α blood levels of older individuals, as observed in humans (Bruunsgaard et al., 1999). Our study shows correlation between IL-1β and IL-6 production previously found in LPS-stimulated human monocytes (Bruunsgaard et al., 1999), that can be explained by the stimulatory effect of IL-1β on IL-6 production (Abbas and Lichtman, 2003). On the other hand, IL-6 is known to both suppress IL-1β and TNF-α production and block the effect of IL-1β by inducing the production of IL-1 receptor antagonist (Schindler et al., 1990; Jordan et al., 1995). This might explain why there was no obvious increase in TNF-α production in the older cats. Furthermore, by upregulating IL-6, IL-1β could actually inhibit the upregulation of TNF-α, a cytokine with far wider effects than IL-1β itself (Abbas and Lichtman, 2003), and thereby limit its own harmful effects in aged individuals (Schindler et al., 1990; Jordan et al., 1995). The age-related decline in IL-10 transcription, however, might foster IL-12 p40 production due to lack of inhibition (Abbas and Lichtman, 2003).

Our findings show that circulating monocytes might be responsible for some of the immune dysfunctions seen with increasing age, particularly 'inflamm-aging'. There is evidence for a progressive activation of monocytes with age, compatible with the previously postulated 'macrophaging' (Franceschi et al., 2000). Since this progressive inflammatory status appears to occur in both ill and healthy older individuals, then it can be regarded as a characteristic of both successful and unsuccessful aging (Franceschi et al., 1999; 2000). Upregulation of IL-1β, IL-6 and IL-12 might represent adaptation of the immune system to ensure its capacity to deal with infectious agents (Abbas and Lichtman, 2003). In the case of humans and non-barrier-maintained animals, it might also be the consequence of the successful adaptation to stresses which occurred throughout an individual's life (Franceschi et al., 2000).

Previous studies on activated monocytes/macrophages from several species are equivocal. For example, aged mice have exhibited decreased IL-1, TNF-α, IL-6 and nitric oxide production (Effros et al., 1991; Wallace et al., 1995), whereas monocytes from aged humans exhibited significantly lower IL-1β, but increased IL-6 and IL-10 production after stimulation (Sadeghi et al., 1999). In aged mice and rats, reduced MHC class II and monocyte chemotactic protein-1 transcription and translation, respectively, was observed (Herrero et al., 2002; Reale et al., 2003). In another study higher phagocytic capacity after LPS treatment, despite lower phagocytosis levels without activation was described in rat macrophages (Tasat et al., 2003). In conjunction with the present study, these findings suggest an imbalanced state of monocyte/macrophage function and thereby the innate immune response with age. They also indicate that by down-regulating certain constitutive levels of stimulatory functions such as MHC II expression, monocytes may counteract and balance their otherwise increasingly pro-inflammatory state in aging individuals (Franceschi et al., 2000).

With regard to the cat, further studies on the cytokine transcription of activated feline monocytes are needed to explain why FIV infection induces a less intense immune reaction in aged animals and why older cats are less affected by FIP (George et al., 1993; Rohrbach et al., 2001). It seems plausible that despite increased or similar constitutive cytokine transcription, monocytes of older cats might exhibit an impaired reactive activation after virus infection (Kipar et al., 2005).

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