

CLINICAL STUDY

The relationship between cell surface markers, cytokines, ageing, and cigarette smoking

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Abstract: The purpose of this study was to investigate the modulation of selected cell surface markers and proinflammatory cytokines production in relation to ageing, and cigarette smoking. The analysis of cell surface receptors was performed by the flow cytometry and cytokines levels were evaluated by the sandwich enzyme immunoassays. We found a decreased expression of CD69, CD28, CD11b, CD95 markers in old population compared to young people ($p < 0.05$; $p < 0.001$). The memory CD45RO lymphocytes were markedly expanded in older population in comparison to young donors ($12.93 \pm 5.92\%$, $p < 0.001$) and the selectin CD62L was significantly increased on granulocytes in aged people ($p < 0.05$). Our findings demonstrated an augmented level of CD3 and CD28 on lymphocytes in smokers ($p < 0.05$; $p < 0.005$). The significant depression of CD16+56 molecule was recorded in smokers ($10.86 \pm 0.80\%$) when compared to non-smokers (14.44 ± 0.46 ; $p < 0.05$). Our results showed a significantly diminished levels of interleukin (IL)-1 β (1.93 ± 0.48 pg/ml), and increased levels of IL-6 and tumor necrosis factor (TNF)- α in elderly population compared to young people ($p < 0.05$; $p < 0.001$). The present data support previous suggestions that senescence and cigarette smoking may contribute to changes in the immune system activity, resulting in altered cell surface marker expression and cytokine levels (Tab. 1, Fig. 3, Ref. 81). Full Text (Free, PDF) www.bmj.sk.

Key words: immune system, ageing, cell surface markers, cytokines, smoking.

Abbreviations: FITC – fluorescein isothiocyanate, IL – interleukin, NK – natural killer, PE – phycoerythrin, TNF – tumor necrosis factor.

Immunosenescence is an age associated decline of immunity involving multiorgan changes. As the mean age of the population increases, an increase of diverse pathologies associated with the immunosenescence has been observed in the developed countries. Changes in the cellular components of the immune system associated with ageing contribute to an increased incidence and severity of infectious diseases and possibly cancer in the elderly (Tarazona et al, 2002; Pawelec, 2006; Vasto et al, 2007). Ageing is due to complex interaction of genetic, epigenetic, and environmental factors. Among the numerous theories that explain the process of ageing, the mitochondrial theory of ageing states that oxidative damage, generation of reactive oxy-

gen species, is a major determinant of ageing (Capri et al, 2006a; Alexeyev et al, 2004). The theory of ageing evolution postulates that senescence is the late deleterious effect of genes that are beneficial in early life (Kirkwood and Austad, 2000; Kirkwood, 2002; Giunta, 2006).

Human ageing is accompanied by a slight lymphopenia and a decline in immune functions. The ageing leads to the replacement of naive T cells expressing CD45RA⁺ on the surface by memory T cells expressing CD45RO⁺. Accumulation of CD28-CD8⁺ T cells that are defective in response to antigenic stimulation is a hallmark of age associated decline in T cell function. Human ageing is associated with many physiological and cellular changes, and cellular activation markers, many of which are due to alterations in plasma membrane functioning. Several studies have shown an increased expression of CD95 on lymphocytes from elderly donors, which could be due to age-related changes in the susceptibility of these cells to apoptosis (Benanou et al, 1998; Fagnoni et al, 2000; Pinti et al, 2002). Bryl et al (2001) demonstrated a decreased level of CD95 that may confer some level of protection from an early onset of apoptosis, before activated lymphocyte completes its task, or it may be an indication of an decreased ability of the immune system to eliminate „used“ T cells at the end of an immune response, especially in elderly. A similar antiapoptotic mechanism that involves inhibition of CD95-mediated apoptosis could exist also in smoking (Suzuki et al, 1999; Briggs et al, 2002; Imirzalioglu et al, 2005).

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Several experimental work suggest a bidirectional interaction between ageing and cigarette smoke, decrease of telomerase activity, triggering of inflammation, challenges in organ maintenance and repair (Tsuji et al, 2004; Tudor, 2006; Morla et al, 2006). The use of nicotine-containing tobacco has been associated with immunomodulation and an increase of specific diseases, such as respiratory tract infections, chronic airway disease, asthma, allergies, and lung and other cancers. Older people who smoke are more susceptible to vascular and lung diseases including lung cancer than aged-matched non-smokers (Hallquist et al, 1999). Nicotine alone or exposure to cigarette smoke has been shown to alter immune responses by decreasing inflammation, decreasing the antibody-forming cell response of splenocytes, decreasing T cell receptor-mediated signalling, decreasing proliferation of peripheral blood mononuclear cells, and suppressing natural killer (NK) cell activation (McAllister-Sistilli et al, 1998; Sopori et al, 1998; Mellon et al, 1999; Kalra et al, 2000; Lu et al, 2007).

Cellular components of both adaptive and innate immune systems produce different chemokines and cytokines, involved in different signalling pathways among cells, and modulate the effector function during immune response, playing a key role in elderly. Several writers have reported an altered production of cytokines, including interleukin (IL)-6, tumor necrosis factor (TNF)- α , IL-1, IL-10, IL-4, playing a direct role in the pathogenesis of age-related disorders, but the emerging data of cytokine changes in human studies are not consistent. Inflammation is the leading force driving immunosenescence and the chronic low-grade inflammatory state characteristic for ageing represents the predisposing substrate on which age-related diseases, such as atherosclerosis, Alzheimer's disease, diabetes, autoimmune disorders, cardiovascular diseases, cancer, asthma, infectious diseases and osteoporosis, might emerge (Wick et al, 2003; Licastro et al, 2005; Ginaldi et al, 2005; Il'yasova et al, 2005; Bernstein and Murasko, 2006; Olivieri et al, 2006; Li et al, 2006; Vasto et al, 2007; Horner and Strunk, 2007; Corrales et al, 2007).

Our aim was to investigate age- and smoke-dependent changes in the expression pattern of the cell surface markers on leukocytes which might contribute to the remodelling of the immune system in elderly and smoker adults among Slovak population. Furthermore, we evaluated the circulating concentration of proinflammatory cytokines IL-1, IL-6 and TNF- α , in order to highlight possible differences in the synthesis of these factors during the senescence and smoking.

Material and methods

Study population

The immune parameters were examined in 140 aged people (range 60–65 years) and 150 young people (range 20–25 years). The investigated subjects were relatively healthy volunteers without any acute disease, diabetes or dementia, the scoring of minimal state examination was more than 24, the body mass index was more than 17, the alcohol consumption levels was less than 50 cl per week for men and 35 cl per week for women. The people who never smoked were defined as non-smokers (n=214)

and current regular smokers (1 or more number of cigarettes per day) as smokers (n=76). The study had the approval of the local ethics committee and written informed consent was obtained from all participants.

Immunophenotyping using flow cytometry

Anticoagulated whole blood cells were stained with fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies anti-CD69, anti-CD62L (Becton Dickinson), anti-CD28 (Beckman Coulter), and phycoerythrin (PE) conjugated anti-CD11b, anti-CD45RO (Becton Dickinson), anti-CD152 and anti-CD95 (Beckman Coulter). The antibodies CD3-FITC/CD16+56-PE, CD8-FITC/CD4-PE/CD3-ECD, CD19-PC7, anti-HLADR-PC5 (Beckman Coulter) were used for multiple colour staining. The analysis of cell surface antigens was performed within 24 h and a minimum of 10,000 events for each sample was collected in the list mode. Erythrocytes were lysed with the lysing solution OptiLyse C (Beckman Coulter). Flow cytometric analyses were performed on the Cytomics FC 500 (Beckman Coulter) using CXP (Beckman Coulter) software.

Interleukin detection

The levels of proinflammatory cytokines IL-1 β , IL-6 and TNF- α were measured in human serum samples by the commercially available sandwich enzyme immunoassays Quantikine (R&D Systems). The range assay for IL-1 β , IL-6 and TNF- α was 3.9–250 pg/ml, 3.12–300 pg/ml and 5.6–1000 pg/ml, respectively. The minimal detectable dose was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

Statistical analysis

The analysis was performed using the SPSS statistical software. Baseline data were compared by an unpaired *t*-test for normally distributed continuous variables. The data are presented as the mean standard error of the mean. Correlation analyses were performed by the Spearman's test. The results were considered significant when the P value was <0.05.

Results

Cell surface antigens detection by flow cytometry

We found a diminished expression of CD69 and CD28 markers when we compared young (2.12 \pm 0.14 % and 29.14 \pm 9.89 %, respectively) and old lymphocytes populations (0.98 \pm 0.07 % and 22.72 \pm 6.43 %, respectively). The basal values of the activation marker CD69 were significantly diminished further on monocytes (p<0.05) and granulocytes (p<0.001) in aged people. Our analysis suggested a significant decrease of CD95+ lymphocytes in elderly (1.25 \pm 0.11 %) when compared to young people (2.13 \pm 0.19 %) (p<0.001). Memory CD45RO lymphocytes were markedly expanded in older population (26.08 \pm 8.84 %) in comparison to young donors (12.93 \pm 5.92 %; p<0.001). The differences occurred likewise in the expression of the cell adhesion

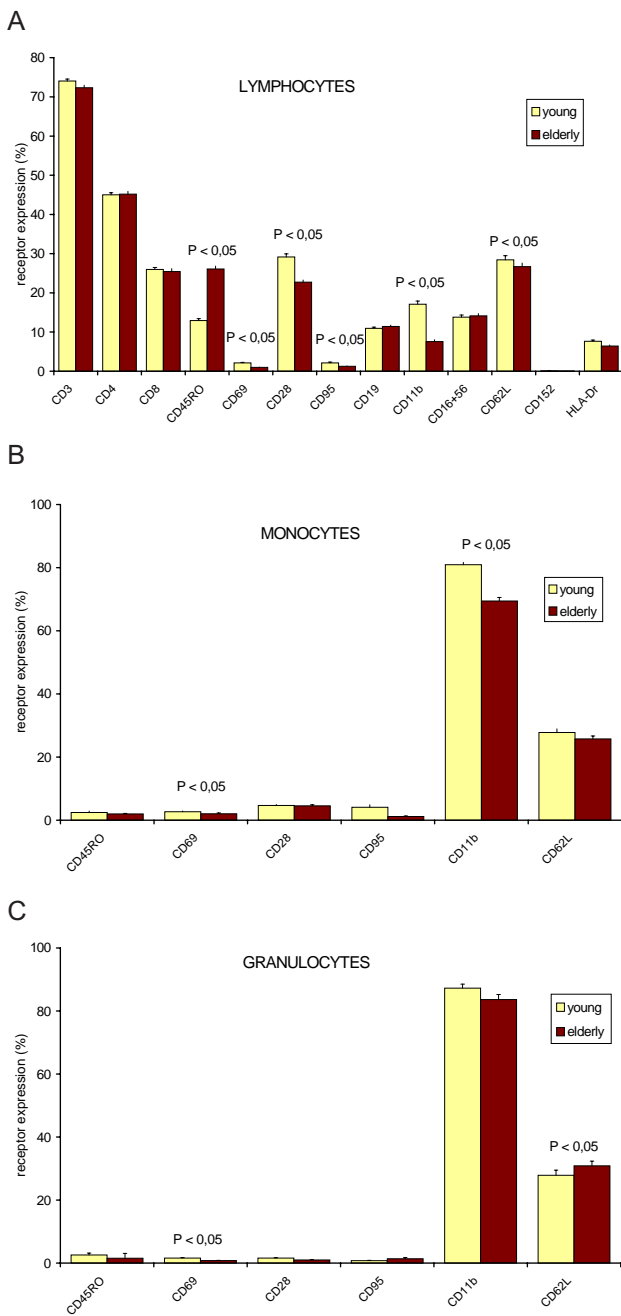


Fig. 1. The cell surface antigen expression in young and aged people. The cell surface marker measurement was performed in elderly (n=140) and young population (n=150) using a flow cytometer. The results p<0.05 were considered significant. A: lymphocytes; B: monocytes and C: granulocytes.

molecules CD11b and CD62L between young and elderly people. The integrin CD11b was diminished on lymphocytes and monocytes (p<0.001) and selectin CD62L was increased on granulocytes (p<0.05) in elderly subjects. No significant differences were found in the CD3, CD4, CD8, CD16+56, HLA-Dr, CD19 and CD152 expression between young and aged people (Fig. 1A, B, C).

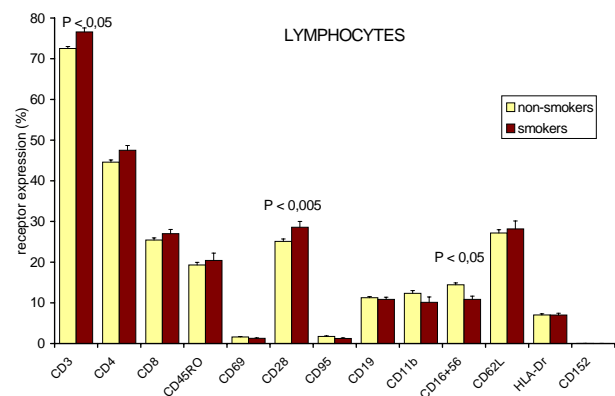


Fig. 2. The lymphocyte cell surface markers expression in smokers (n=76) and non-smokers (n=214). The antigen receptor expression was detected by a flow cytometer in all populations. The results p<0.05 were considered significant.

Figure 2 shows the results of cell surface marker analysis of both, the smokers and non-smokers populations. The percentage of costimulatory molecule CD28 was increased on lymphocytes in the smoker population (28.60±1.09 %, p<0.005). A depressed expression of CD16+56 molecule was recorded in smokers (10.86±0.80 %) when compared to non-smokers (14.44±0.46; p<0.05). The CD3 marker was augmented in the smoker population in comparison to non-smokers (p<0.005 and p<0.05, respectively). No differences were observed in the expression of CD4, CD8, HLA-Dr, CD19, CD69, CD11b, CD62L, CD152 (high affinity receptor for the costimulatory molecules CD80), and CD45RO memory cell marker between smokers and non-smokers. The summary of changes in smokers and elderly is shown in the Table 1.

Interleukin detection

Our results showed a significant decrease of IL-1β in elderly population (1.93±0.48 pg/ml) compared to young people (p<0.05). Levels of IL-6 and TNF-α were increased significantly (p<0.001) in the older group (2.63±0.19 pg/ml and 9.32±3.08 pg/ml) when compared to young population (0.86±0.08 pg/ml and 7.63±1.38 pg/ml) (Fig. 3). The differences in the levels of IL-1β, IL-6 and TNF-α between the smokers and non-smokers were not significant (data not shown). Interestingly, an inverse correlation was found between TNF-α levels and CD62L expression on granulocytes in elderly people (r=-0.17; p<0.05).

Discussion

The immunosenescence is characterised by the three main aspects, the shrinkage of the T cell repertoire and the accumulation of oligoclonal expansions (megaclones) of memory/effector cells directed towards to infectious agents, the involution of the thymus and the exhaustion of naive T cells, and a chronic inflammatory status called inflammaging (Capri et al, 2006 b;

Tab. 1. The summary of changes in smokers and elderly subjects.

| CD marker | Region | Smokers ^a | Elderly subjects ^b |
|-----------|--------------|----------------------|-------------------------------|
| CD69 | lymphocytes | | ↓ |
| | monocytes | | ↓ |
| | granulocytes | | ↓ |
| CD28 | lymphocytes | ↑* | ↓ |
| CD11b | lymphocytes | | ↓ |
| | monocytes | | ↓ |
| CD56+16 | lymphocytes | ↓ | |
| CD3 | lymphocytes | ↑ | |
| CD95 | lymphocytes | | ↓ |
| CD45RO | lymphocytes | | ↑ |
| CD62L | granulocytes | | ↑ |

^a – smokers compared to nonsmokers, ^b – elderly subjects compared to young people, ↑↓ – statistical significant differences ($p < 0.05$) ↑* – statistical significant differences ($p < 0.005$)

Giunta, 2006). The decline in immune function with an increasing age leads to an impaired responses to vaccination, an increased incidence of autoimmune disorders and increased morbidity and mortality to infectious disease. The thymus is a central lymphoid organ responsible for the production of naive T cells, which play a vital role in mediating both cellular and humoral immunity. Chronic involution of the thymus gland is thought to be one of the major factors contributing to the loss of immune function with an increasing age (Gruver et al, 2007). An increased number of memory cells results in a reduced ability to respond to new antigens. Age-associated alterations in the proportion of T cell subsets have been well documented in humans. There is clearly a higher number of CD45RO+ memory phenotype cells and fewer CD45RA+ naive phenotype cells. Our findings, the accumulation of CD45RO+ T cells, are consistent with the previous reports (Herndon et al, 1997; Neuber et al, 2003).

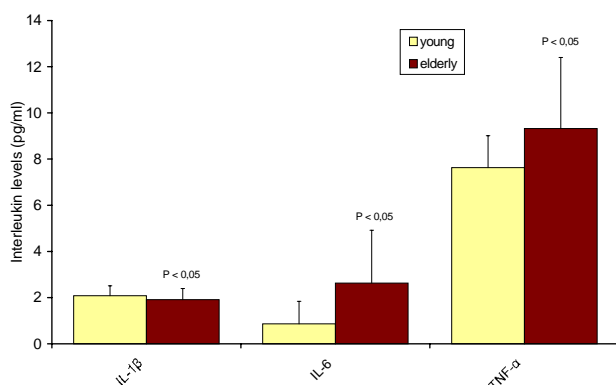


Fig. 3. The interleukin levels in young and elderly people. The levels of proinflammatory cytokines IL-1 β , IL-6 and TNF- α were measured in human serum samples by sandwich enzyme immunoassays. Data were compared by an unpaired t-test for normally distributed continuous variables. $p < 0.05$ were considered significant.

Cigarette smoking causes many life-threatening diseases, including lung cancer, colon cancer, emphysema, and heart disease. Smoking has a long-term chronic effect on many important aspects of the inflammatory and immune responses and may decline the innate and acquired immunity. Many *in vitro* and *in vivo* studies gave evidence that tobacco smoking affect both cell-mediated immunity and humoral immunity, including cytokine and immunoglobulin production, chemotaxis, phagocytosis, immune surveillance, and others (Mooney et al, 2001; Moszczynski et al, 2001; Zeidel et al, 2002; Thompson-Cree et al, 2004; Palmer et al, 2005; Wetzel et al, 2007).

The findings of this study demonstrated an augmented level of CD3 lymphocytes in smokers. Others investigators have reported that increased numbers of CD3+ T cells, as well as CD4+ and CD8+ T cells, and elevated T cell responsiveness was associated with smoking (Schaberg et al, 1997; Tanigawa et al, 1998; Loos et al, 2004). Our data are consistent with previous studies that demonstrated a decreased NK cells number in smokers (Jung and Irwin, 1999; Moszczynski et al, 2001; Lu et al, 2007). Important changes occurring on the cell surface during the senescence contribute to the complex remodelling of the immune function, the activation-related antigen CD69 might influence the signal transduction and the resolution of inflammatory immune responses. We observed a decreased cell surface expression of CD69 in elderly subjects, what may be the first signal of the impairment of arly activation, but it is inevitable to confirm this hypothesis *in vitro*. The altered regulation of CD69 correlate with the broken immunological tolerance that is associated with some disease, such as cancer, autoimmune diseases (Fulop et al, 2003; Provinciali and Smorlesi, 2004). During studies we observed that the lymphocyte proliferation activity was significantly increased in smokers and decreased in aged population in comparison to non-smokers and young people (Tulinska et al, 2006). CD152 is either engaged in the downregulation of T cell activation or affected during ageing and nicotine exposure (Zhang and Petro, 1996; Carreno et al, 2000; Leng et al, 2002; Trzonkowski et al, 2002). Conversely, we have demonstrated some alterations of the CD152 cell surface level in elderly and smoker donors.

The phagocytic ability of peripheral blood neutrophils from elderly donors has been shown to be impaired, which may be partially explained by the age-associated reduction in the expression of CD16 (Butcher et al, 2001; Chidrawar et al, 2006). We demonstrated no alterations of CD16+56+ cells in elderly people, and the deterioration effect of smoking on the CD16+56 expression levels, however without depression of phagocytosis (data not shown). The CD11b expression on leukocytes may not be consistent throughout life and the ideas of its upregulation or not affection during ageing are very various (Noble et al, 1999; Armstrong et al, 2001). Our findings, the lower levels of CD11b on lymphocytes in the elderly group, represent a downregulation and protection against excess lymphocyte activation within the vascular system and therefore, may provide a compensatory mechanism for a successful ageing. On the contrary, CD11b+ granulocytes were increased in the subgroup of occasionally smokers (data not shown), and this phenomenon could be asso-

ciated with the priming and/or activation of neutrophils. The presence of chronically activated neutrophils in smokers is relevant to an increased granulocyte aggregability that predisposes to microvascular occlusion and damage, and aggravation of tissue destructive inflammatory diseases. Some investigators have reported a decrease of CD62L expression in peripheral blood cells from elderly subjects compared to young donors, on the other hand, other authors have reported an increased level of CD62L+ lymphocytes (Ginaldi et al, 2000; De Martini et al, 2000; Collazioli et al, 2004; De Martinis et al, 2004; Peres et al, 2004). Our results suggest the overexpression of CD62L on granulocytes from elderly donors, which is interpreted as a compensatory mechanism for a decreased responsiveness and a higher requirement for activation signals rather than an age-related anomaly. This phenomenon may also represent an important mechanism to ensure the first line of response to acute inflammatory stimuli and prevent aged people from an increased susceptibility to acute infections.

Whilst CD28-mediated signalling is increasingly associated with survival, ligation of alternative T cell antigens, such as CD95 (Fas), can trigger apoptosis. The T cell response following an antigen engagement may therefore be influenced by the relative expression levels of these co-receptors as well as by the availability of their ligands (CD80/86 and Fas-L) (Walker et al, 1998). The CD28-deficient cells were more susceptible to CD95-mediated apoptosis (Dennett et al, 2002; Jones et al, 2002). We demonstrated a downregulation of CD28 expression concomitant with the CD95 suppression in aged population, this ensure the comeback to standard survival level. On contrary, the existing CD28 upregulation in smokers contribute to the impairment of apoptotic programme and escalation of the cancer diseases formation. The weakened apoptosis may reduce the death from ischemic diseases of the heart, however may escalate the risk of cancer diseases or lead to accumulation of dysfunctional and self-reactive cells in elderly people, eventually smokers.

Cytokines play a key role during ageing acting in the regulatory communications among cells and the effectors activity during immune response (Alberti et al, 2006). Ageing is associated with a low-grade increase in circulating levels of TNF- α and IL-6 (Bruunsgaard et al, 2002). The results of our study showed that the level of IL-6 and TNF- α but not IL-1 was increased in the elderly people compared to young subjects. A significant increase of IL-6, TNF- α and IL-1 level was found by other authors (Fagiolo et al, 1993; Dirks and Leeuwenburgh, 2006; Mariani et al, 2006). The circulating levels of IL-6 seem to be a strong risk factor for a frailty in elderly, which reflect its association with an increased production of TNF- α . IL-6 and TNF- α is regarded as a sensitive marker of systemic inflammation (Bruunsgaard and Pedersen, 2003). The shift towards an activated immune profile has been hypothesized as an important risk factor in chronic inflammation and the immune system remodelling characteristic for ageing. The role in the pathogenesis of these inflammatory diseases is played by phagocytic cells and the capacity of TNF- α to prime and/or activate phagocytic cells and defend the host against infections (Khawaja et al, 1992; Li et al, 1996; Trinchieri et al, 2004; Capsoni et al, 2005). Our findings suggest the continuity between an el-

evated level of TNF- α and increased phagocytic function in elderly (data not shown). Interestingly, recent study proclaim a negative correlation between TNF- α level and CD62L expression on granulocytes in elderly, that is interpreted as the shedding of CD62L+ granulocytes upon the enhanced activation effect of TNF- α .

Cigarette smoking is generally believed to be responsible for a substantial number of health problems. Smoking causes activation of resident cells and the recruitment of inflammatory cells into the lungs, which leads to the release of pro-inflammatory cytokines, chemotactic factors, oxygen radicals and proteases (Tappia et al, 1995; Karimi et al, 2006). Some authors suggested that smoking may skew the immune system toward Th2 pattern (Cozen et al, 2004; Phaybouth et al, 2005; Vassallo et al, 2005). Other study showed a suppressive effect of smoking on the number of eosinophils in human blood. This may be a reflection of local shifts in Th1/Th2 cytokine balance or an anti-inflammatory effect of substances in the smoke (van der Vaart et al, 2004). Lambert et al (2005) have shown that cigarette smoke extracts inhibit IL-2, IFN- γ , and TNF- α production in stimulated lymphocytes obtained from the peripheral blood. Our results revealed that IL-6 and TNF- α levels increased in smokers compared to non-smokers, but this trend was not significant.

The inflammatory scenario characterises most of the age associated diseases, generation of reactive oxygen species causing both oxidative damage and eliciting an amplification of the cytokines release, thus perpetuating a vicious cycle resulting in a chronic systemic proinflammatory state. The tissue injury and healing mechanisms proceed simultaneously over decades and is a major determinant both of the ageing process and the development of the age associated diseases. Presently, in majority of developed countries, the human lifespan is markedly increased, and many individuals are living into postreproductive senescence, an evolutionarily naive life epoch (Vasto and Caruso, 2004; Lupien and Wan, 2004; Ginaldi et al, 2005).

In conclusion, our data suggest that the CD69, CD28, CD11b, CD95 markers within peripheral blood decreases with age. The memory CD45RO lymphocytes and CD62L+ granulocytes were markedly expanded in elderly population. The cigarette smoking resulted in the augmentation of CD3+ and CD28+ lymphocytes and downregulation of CD16+56+ cells. We have shown that levels of IL-1 β were diminished and IL-6 and TNF- α increased during the senescence. The TNF- α level decreased linearly with an increasing expression of CD62L on granulocytes among elderly people.

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