Serum from aged F344 rats conditions the activation of young macrophages

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Abstract

There is considerable controversy about the molecular mechanisms responsible for the variations in innate immunity associated with age. While in vivo, aged animals and humans react to an inflammatory signal with an excessive production of pro-inflammatory cytokines, studies in vitro generally show that this response is attenuated in macrophages from old individuals. In an effort to examine possible extrinsic factors that might affect the response of macrophages to lipopolysaccharide (LPS), we have challenged peritoneal macrophages obtained from young rats with sera obtained from rats of different ages. Our results indicate that the serum from aged rats significantly impairs the capacity of young macrophages to induce tumor necrosis factor-alpha (TNF-α) production, while at the same time it increases the basal levels of interleukin-6 (IL-6). The effect of serum from aged donors on TNF-α secretion requires pre-incubation and is sensitive to heat inactivation. In contrast, the stimulating effect on IL-6 is resistant to heat, and thus should not be due to a protein factor. Therefore, our results indicate that the age-related changes in macrophage activity are not only the consequence of intrinsic changes, but there also appears to be a modulatory effect imparted by the external milieu.

Keywords: Aging; Macrophages; Cytokines; Lipopolysaccharide; TNF-α; IL-6

1. Introduction

Aging is characterized by a decline in both innate and adaptive immunological functions. In the case of lymphocytes, there is considerable coherence between the defects found in vivo and those described using isolated cells in vitro. For example, in vitro studies have shown that the proliferative capacity of lymphocytes is generally attenuated during aging (DeVeale et al., 2004), and a similar behavior could be extrapolated from the decreased response to influenza vaccination observed in old individuals, in vivo (Boon et al., 2002; Sambhara et al., 2001; Webster, 2000). In contrast, there is considerable discordance between in vivo and in vitro data in relation to age-related changes in macrophage activation. This is exemplified by the production of pro-inflammatory cytokines. Most in vivo studies indicate that aged individuals have an increased basal level of serum interleukin-6 (IL-6) and other pro-inflammatory cytokines (Franceschi et al., 2000). In addition, production of cytokines in vivo in response to external stimuli, such as lipopolysaccharide (LPS) has also been found to be strongly exacerbated in aged individuals (primarily rodents). It is generally believed that macrophages represent a central source of these molecules during inflammatory episodes. However, the majority of studies done with isolated macrophages incubated in vitro indicate a

Abbreviations: DC, dendritic cells; FBS, fetal bovine serum; IL, interleukin; LPS, lipopolysaccharide; NO, nitric oxide; TLR, toll like receptors; TNF-α, tumor necrosis factor-alpha

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dramatic age-related decline in the secretion of pro-inflammatory cytokines and chemokines (Gomez et al., 2005; Plackett et al., 2004; Plowden et al., 2004). For example, in macrophages from aged mice, TLR stimulation resulted in the secretion of substantially lower levels of both pro-inflammatory cytokines (tumor necrosis factor-alpha, TNF-α, IL-6) compared to younger counterparts (Renshaw et al., 2002). Similarly, resident naïve peritoneal macrophages isolated form aged mice exhibit diminished expression of TNF-α, IL-10, IL-6 and IL-1β in response to LPS, compared to young control mice (Vega et al., 2004). Comparable discrepancies between in vivo and in vitro results have been obtained using other cells of the immune system, such as dendritic cells (DC). Dendritic cells have long been considered as a crucial nexus between the innate and the adaptive responses (Degli-Esposti and Smyth, 2005). It has been observed that in vitro generated DCs, derived from monocytes obtained from animals of different ages show no age-associated differences in activation, in spite of the fact that the original monocytes do show several age-related changes in activity (Lung et al., 2000; Saurwein-Teissl et al., 1998).

Taken together, the observations cited above indicate that the effect of age on macrophage activation cannot be recapitulated in vitro, using isolated macrophages. A possible explanation for this discrepancy might be a difference in the extracellular milieu surrounding the macrophages. In other words, the age-related defects could result from a compound process, affecting both the 'seeds' (macrophages) and the 'soil' (extracellular milieu). As an example, in the vascular system, the reduced re-endothelization in response to injury observed in aged individuals is due to lower serum levels of VEGF, rather than intrinsic cellular defects in endothelial cells (Gennaro et al., 2003). Similarly, passive transfer of T cells from aged to young animals leads to a senescent phenotype in the B cells of the recipient. This effect seems to be due to modulation of the B cells by the T cells, rather than intrinsic defects on the B cells (Miller, 1999).

In this study, we designed experiments to test whether factors present in the serum could explain the conflicting results observed between in vivo and in vitro experiments, with respect to cytokine production by macrophages during aging. For this, we cultured peritoneal macrophages from young rats in the presence of serum obtained from adult, middle-aged or senescent rats. Our results indicate that in macrophages from young rats, the serum from old rats induces a rapid increase in the basal expression of IL-6, as has been observed during normal aging in vivo. In contrast, we observed that pre-incubation of macrophages from young rats in the presence of serum from old rats leads to a lower induction of TNF-α in response to a challenge with LPS. In conjunction with data from the literature, this suggests that the increased induction of this cytokine in vivo is not the result of either intrinsic or extrinsic factors affecting macrophage activity. In light of these findings, we propose that in vitro studies of aged cells should be revisited, taking into consideration the possible effects that the age-specific external milieu exerts on cell activation.

2. Materials and methods

2.1. Cell culture

Young (3-month-old) male Fischer 344 rats were injected intraperitoneally with 20 ml of 4% (w/v) thioglycolate broth (Difco, Detroit, MI, USA) and were sacrificed by decapitation 4 days after the injection. Peritoneal macrophages were collected by washing with phosphate buffered saline (PBS). Cells were counted using neutral red dye, adjusted to 2×10^6 cells/ml and seeded in 96-well plates in RPMI-1640 medium supplemented with 5% fetal bovine serum, 5% horse serum. After 2 h at 37 °C, 5% CO_2, non-adherent cells were discarded by medium aspiration and the attached cells were supplemented with medium containing either FBS or the appropriate rat serum, as indicated.

2.2. Animals

Male Fischer 344 rats of different ages were obtained from the National Institute on Aging (NIA). Animals were fed NIH31 diet ad libitum, and they were housed individually in a specific pathogen-free facility at the Lankenau Institute for Medical Research. These rats have a median lifespan of 24 months and were sacrificed at the ages of 6 months (adult), 15 months (middle-aged) or 22–24 months (old). Animals were sacrificed by decapitation, and blood was allowed to coagulate for 10 min at room temperature before obtaining serum by centrifugation. Aliquots of serum were immediately frozen in liquid nitrogen until use. The serum from each individual animal was evaluated for the presence of both inflammatory cytokines and acute phase proteins, using commercially available enzyme-linked immunosorbent assay (ELISA) kits and western blots, respectively. Sera that were free of inflammatory markers (the vast majority) were pooled using at least three different animals for each pool. For some experiments, serum was heat inactivated at 50 °C for 30 min. Before addition to the cells, RPMI medium was supplemented with rat serum to a final concentration of 10% and filtered through a 0.33 μm filter. Unless otherwise indicated, cells were challenged 24 h later with 100 ng/ml LPS (serotype 055:B5, Sigma Chemical Co., St. Louis, MO, USA). Either 24 or 48 h later, the conditioned medium was collected, spun at $14{,}000 \times g$ and stored at −80 °C.

2.3. Quantitative determination of cytokines and chemokines

TNF-α, IL-6, IL-10, monocyte chemotactic protein 1 (MCP-1) and macrophage inflammatory protein 1-alpha (MIP-1α), either in serum or in the conditioned media, were evaluated using commercial ELISA kits as recommended by the manufacturer. For TNF-α, IL-6, IL-10 and MCP-1, we used rat specific OPTiEA kits (Cat. Nos.: 2697KI, 2705KI, 2611KI and 2610KI, respectively, BD Pharmingen, San Diego, CA, USA). MIP-1α was analyzed using Quantikine® M ELISA kit (MMA00, R&D Systems Minneapolis, MS, USA). This is a murine kit, which has been successfully used for detecting rat MIP-1α (Barnes et al., 1998). The levels of detection of the kits are 31.3 pg/ml for TNF-α 78 pg/ml for IL-6, 15.6 pg/ml for IL-10, 31.3 pg/ml for MCP-1 and 75 pg/ml for MIP-1α.

2.4. Data acquisition and processing

Statistic analyses were done using Graph Pad software, Version 3.02 for Windows (GraphPad Software, San Diego, CA). Comparisons between treatments were evaluated using the non-parametric test of Mann–Whitney, correlations were analyzed by the Fischer test, and curves were analyzed with the Friedman and Quade test.

3. Results

3.1. Serum from aged rats inhibits TNF-α secretion by young macrophages

To establish the effect of extrinsic factors on macrophage activation, we collected serum from adult (6 months), middle-

aged (15 months) and old (22–24 months) rats, and pools of sera derived from at least three animals of each age group were used to incubate peritoneal macrophages obtained from young animals. The next day, cultures were induced for an additional 24 h with LPS and TNF-α secretion was measured in the supernatants. In all cases, LPS treatment of young macrophages led to a strong induction of TNF-α production. However, this induction was approximately 40% lower ($p < 0.05$) when macrophages were pre-incubated with serum from aged rats, as compared with serum from rats from either the adult or middle-aged groups (Fig. 1). In these experiments, the presence of rat serum by itself did not induce detectable levels of TNF-α in the conditioned medium (data not shown). We have repeated this experiment with three different sets of serum from each age group, prepared at different times and tested each preparation with independently isolated macrophages and obtained similar results. Even though induction of TNF-α production was best in the presence of serum from adult rats, it should be noted that this was about five-fold less than in the presence of FBS (0.4–0.6 ng/ml, compared to 2–3 ng/ml). As an additional control, the serum from each individual animal had been previously evaluated for a panel of inflammatory markers. Serum levels of TNF-α, IL-6, IL-10 and MIP-1α were all below the detection level of the respective assays in sera from animals of all ages (data not shown). In the case of MCP-1, its levels were detectable in all groups (156.2 ± 94.2, 163.0 ± 92.7 and 157.8 ± 83.2 μg/ml for adult, middle-aged and aged, respectively), but did not show age-related differences. Furthermore, we did not detect acute phase proteins (alpha-1 acid glycoprotein (AGP) or haptoglobin) that could indicate a chronic inflammatory process in any of the animals, which may affect the effect of the serum on macrophages. Regarding other indicators of immune function, we did not detect basal proliferation of splenocytes or induction of the mRNAs for IL-2, INFγ or CD-25 (data not shown).

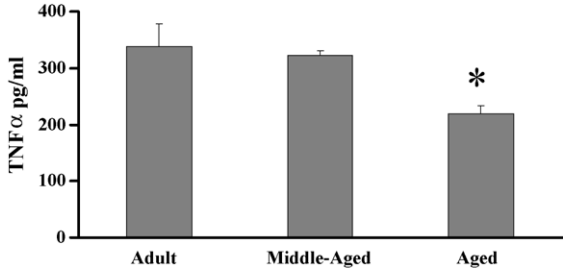

Fig. 1. TNF-α induction is reduced in the presence of serum from old rats. Peritoneal macrophages from young F344 rats were incubated for 24 h in medium supplemented with 10% rat serum, and stimulated for an additional 24 h with 100 ng/ml LPS. Conditioned media were tested for TNF-α by ELISA. The values are from cultures of macrophages generated from five individual young animals, incubated with serum from pools of three animals per age (adult, middle-aged and old). The results correspond to average ± S.E.M. The asterisk denotes statistical significance ($p < 0.05$). Basal levels of TNF-α in the absence of LPS were undetectable under all conditions tested.

3.2. The effect of serum on TNF-α production is due to a titratable factor present in aged serum

Our observations could be explained either by lack of an essential factor (such as lipopolysaccharide binding protein (LBP), for example) or the presence of an inhibitory factor in the serum from aged rats. To evaluate this, we pre-incubated the LPS in the presence of FBS, which is fully competent in supporting the activating effect of LPS, for 30 min at 37 °C before mixing with the rat serum (from animals of different ages) and addition to the cells. This pre-treatment did not abolish the age-related differences observed in Fig. 1 (see Fig. 2A). The effect we have observed is dose-dependent, since dilution of the young serum with serum from aged animals (but keeping the total rat serum concentration constant at 10%) results in a progressive loss of inducibility (Fig. 2B). Therefore, we conclude that the lower induction of TNF-α observed in the presence of serum from old rats is due to the presence of an inhibitory factor, rather than lack of an essential activator.

3.3. The factor present in aged rat serum is resistant to thermal inactivation

To analyze some of the physical characteristics of the factor in serum from aged rats, which suppresses TNF-α production, we first established its stability as a function of time in culture medium. Fig. 3 indicates that the age-related difference was

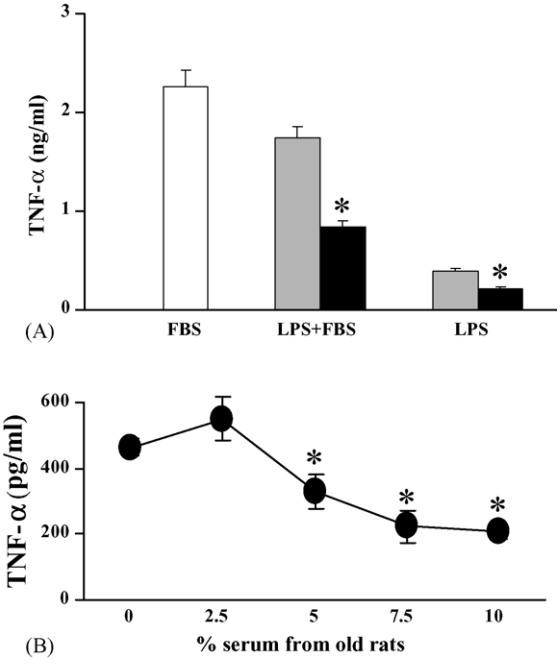

Fig. 2. The reduced TNF-α production is not due to lack of a positive factor. (A) Cells were cultured for 24 h either in FBS (white bar) or in the presence of serum derived from either adult (gray bars) or old (black bars) rats. At the end of this period, all cells were stimulated for 24 h with LPS, either directly (bars labeled FBS or LPS) or with LPS that was previously pre-incubated with 50% FBS (bars labeled LPS + FBS). TNF-α production was then measured in the culture medium. The asterisks denote statistical significance ($p < 0.05$). (B) The effect of mixing young and old serum on TNF-α production after 24 h was evaluated.

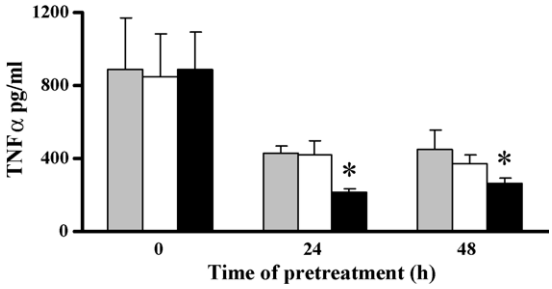

Fig. 3. The inhibitory effect is stable, and requires pre-incubation. Cells were cultured as before, but induction with LPS was done either at the same time as serum addition (time 0), or 24 or 48 h later. Cultures were kept for a further 24 h and TNF-α was evaluated in the conditioned media. Gray bars: adult serum; white bars: middle-aged serum; black bars: old serum. The asterisks denote statistical significance ($p < 0.05$).

maintained for at least 48 h, indicating at least partial stability of the factor(s) in culture medium. The decreased induction observed at 24 and 48 h, compared to 0 h, is not specific to aged rat serum, since a similar effect was observed in the presence of FBS (data not shown). Furthermore, the data also indicate that the age-dependent effect of serum on TNF-α production requires pre-incubation, since no difference in induction was observed when cells were treated with LPS and serum simultaneously.

We then tested whether the factor in question is heat stable. For this, sera were heated under the conditions normally used for complement inactivation (50 °C for 30 min). Heat inactivation reduced the amount of TNF-α released by macrophages in the presence of serum from adult and middle-aged animals, but not in the presence of serum from old rats (Fig. 4). As a result, heat inactivation resulted in elimination of the age-related difference in induction. These results suggest that, in addition to the stable inhibitory factor present in the serum of aged rats, there is also a heat-sensitive activator present in the serum from younger animals. In fact, a similar 35% reduction in TNF-α production was observed when FBS was heat inactivated (data not shown). This indicates that the adult rat serum contains a heat-sensitive activator of TNF-α production, which appears to be missing from the serum of aged rats.

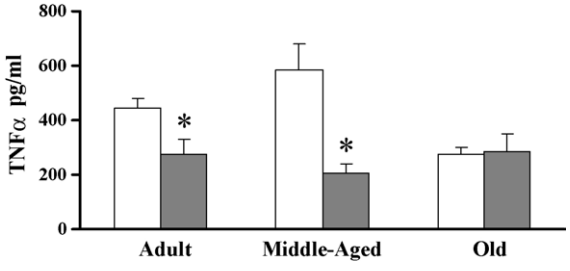

Fig. 4. The serum from old animals is resistant to heat denaturation. Sera were thermally treated before incubation, as described in Section 2, and TNF-α production was evaluated as before. White bars: untreated serum; gray bars: heat-denatured serum. The asterisks denote statistical significance ($p < 0.05$).

3.4. The serum from old rats affects differentially the expression of other cytokines

TNF-α expression can be modulated by other cytokines, including IL-10 and IL-6 (Aderka et al., 1989; Wang et al., 1994). Since IL-10 can inhibit the in vitro expression of TNF-α (de Waal Malefyt et al., 1991; Fiorentino et al., 1991), a differential early induction of this cytokine could explain our observations, even though IL-10 was not initially detected in any of the sera used in these experiments. Furthermore, IL-10 production by young macrophages was not affected by the age of the rats providing serum (data not shown). This result eliminates IL-10 as a possible factor responsible of the effect of serum from old rats on TNF-α production.

Serum levels of IL-6 have often been reported to be increased in old rats (Barrack, 1997; Daynes et al., 1993; Ershler, 1993; Ershler et al., 1993). However, we did not detect IL-6 in the sera used, even when using an ELISA assay which is accurate to 78 pg/ml. IL-6 has been described as having a dual role during inflammation, as it can act as both a pro- or anti-inflammatory agent, depending on the dose and time of exposure (Aderka et al., 1989; Kamimura et al., 2003). Fig. 5 shows that rat serum induces basal secretion of IL-6 (Fig. 5A), even in the absence of LPS, and this effect occurs with serum of any age. No such induction was observed in the presence of FBS (data not shown). Notably, this induction was most pronounced in the presence of serum from old animals and increased as a function of time in culture. Correlation analysis indicated that the basal level of IL-6 secreted by macrophages from young rats cultured in the presence of serum from old rats was higher than that after incubation with serum from adult or middle-aged rats at all times tested. Furthermore, induction of IL-6 in response to LPS (Fig. 6) was also exacerbated in the presence of serum from old rats ($p < 0.05$).

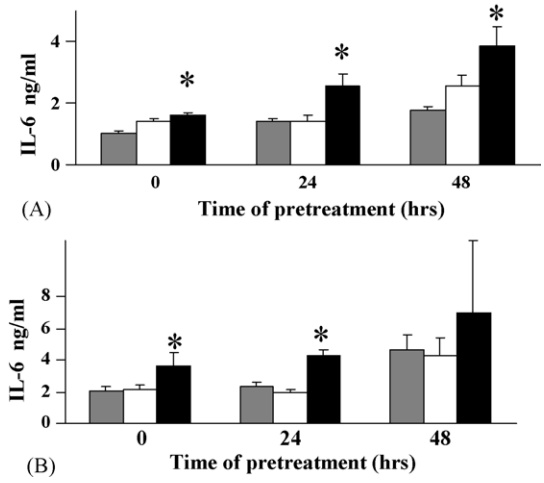

Fig. 5. The serum from old rats induces higher levels of IL-6. Young macrophages were pre incubated for 0, 24 or 48 h in the presence of adult (gray bars), middle-aged (white bars) or old (black bars) rat serum. At the end of this period, further 24 h incubation was carried out, either in the absence (panel A) or in the presence (panel B) of LPS. IL-6 was then measured in the conditioned media. The asterisks denote statistical significance ($p < 0.05$).

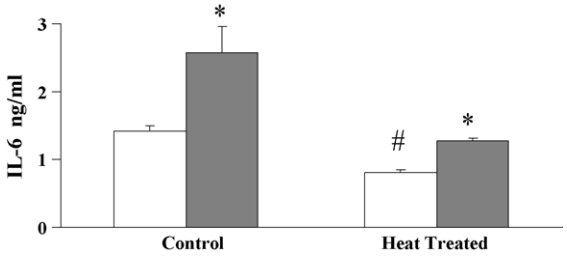

Fig. 6. The induction of IL-6 is resistant to heat. Sera were thermally denatured before incubation, as described in Section 2, and IL-6 production was evaluated as above. White bars: untreated serum; gray bars: heat-denatured serum. The asterisks denote statistical significance ($p < 0.05$).

In contrast to the observations regarding TNF-α, the effect of serum on IL-6 induction did not require pre-incubation, as increased levels of IL-6 were observed even after co-application of the serum and LPS (Fig. 5A). Furthermore, as shown in Fig. 6, IL-6 secretion was induced in the presence of heat-treated serum and the age-related difference was not lost by this treatment (age-dependent ratios for induction are 2.0 ± 0.2 and 1.6 ± 0.2 for control and heat-treated sera, respectively).

4. Discussion

Aging is associated with a progressive decline in immune responsiveness to exogenous antigens. Most studies thus far have focused on cellular alterations, and indeed, defects at several levels, including receptor binding, signal transduction and gene expression have been described (Hawkley and Cacioppo, 2004). Age-related changes in the extracellular/tisular environment have also been suspected to play a role in the observed variations of immune competence during aging (Doria and Frasca, 1994; Stout and Suttles, 2005).

There are many well-described examples that suggest that age-related changes in cell function are not only due to cell-autonomous phenomena, but also to changes in extrinsic factors. For example, by measuring sprouting of vascular microvessels derived from the epididymal fat pad of aged mice, Arthur et al. (1998) have shown that the age-related deficiencies in angiogenesis observed in vivo are likely to be due at least in part to a decrease in angiogenic growth factors present in the serum. Similarly, the effects of caloric restriction on age physiology appear to be at least partially mediated by circulating factors. Indeed, in experiments described by De Cabo et al. (2004), treatment of a macrophage cell line with serum from caloric restricted rats resulted in reduced cell proliferation, enhanced tolerance to oxidative and thermal stress and heightened expression of stress–response genes, as compared to serum from rats fed ad libitum. A similar effect was observed when serum from rats fed ad libitum was supplemented with insulin and insulin growth factor-1 (IGF-1) (De Cabo et al., 2003). In yet another age-relevant model, it has been shown that senescent stromal fibroblasts secrete factors that can disrupt tissue architecture and/or stimulate initiated cells into uncontrolled proliferation. This suggests that, to the extent that they exist in vivo, senescent cells can create a tissue environment that synergizes with oncogenic mutations to promote the progression of age-related cancers (Krtolica and Campisi, 2003).

We have investigated whether a similar effect could be observed in relation to macrophage activation. For this, we established whether changes in serum-borne molecules could be responsible for some of the inconsistencies found in the literature when in vivo and in vitro studies are compared. In the present study we found that, while IL-6 release to the culture medium was increased in the presence of serum from old rats, both under basal and LPS-induced conditions, serum from old rats has a negative effect on LPS-induced TNF-α production. Thus, factors present in the serum from old rats could at least partially explain the age-related increase in IL-6, but in terms of TNF-α induction, the results were divergent from what is observed in vivo, where an exacerbated TNF-α production in response to LPS has been observed (Aderka et al., 1989; Krabbe et al., 2001; Tateda et al., 1996). In addition, we have studied the effects of serum on NO production, and found that, as for IL-6, basal NO secretion is increased up to two-fold by sera from rats of all ages, relative to FBS. Moreover, this increase was most pronounced when serum from old rats was used (unpublished results). Finally, regardless of age, serum had no effect on either IL-10 production or the phagocytic activity of young macrophages (data not shown).

Taken together, our results suggest that the activation properties of macrophages are influenced by specific factors present in the aged milieu. However, since our results did not show a consistent pattern for both IL-6 and TNF-α, we conclude that the dissimilar results are a function of several different factors whose presence or absence affects each parameter independently. We have focused most of our studies on the factors responsible for affecting TNF-α production. From these experiments, we conclude that the serum from old rats contains a stable inhibitory factor. This serum also lacks a heat-sensitive activator present in sera from young rats. However, this same serum is capable of up regulating IL-6 and NO production by macrophages from young rats. While we have ruled out a contribution for most known pro-inflammatory factors, there are many other changes in the serum that occur as animals age. Heat inactivation did not abolish the effect of age on IL-6 and NO induction. However, this same treatment resulted in a loss of the age-related difference in induction of TNF-α. This suggests that the factor responsible for the increased production of IL-6 and NO is not likely to be proteinaceous. This, coupled with the fact that the effect on these mediators does not require pre-incubation, suggests a possible role for advanced glycosylation end products (AGEs), whose serum levels are known to increase with age (Ulrich and Cerami, 2001). Indeed, it has been described that AGEs can induce higher levels of IL-6, TNF-α and NO in macrophages (Chang et al., 2004; Iida et al., 1994; Morohoshi et al., 1995; Rojas et al., 1996). It is, therefore, possible (though admittedly speculative) that AGEs could play a role in our observations, at least with regards to the effects of serum from old rats on IL-6 and NO. In contrast, the factor lacking in old sera and

responsible for the effect on TNF-α is more likely to be a protein, since it is lost from serum from adult rats following heat inactivation. Of course, more complex interpretations have not been formally excluded. For example, even though the initial serum did not contain detectable levels of IL-6, basal expression of this cytokine was noted during the experimental period, even in the absence of LPS. It has been shown that low doses of IL-6 (within the range observed in our studies) can significantly inhibit production of TNF-α by human peripheral monocytes (Aderka et al., 1989). It is thus possible that this early induction of IL-6 could have played a role in the reduced production of TNF-α in response to LPS.

To date, most studies of cytokine production by macrophages from old individuals have focused on possible changes either in CD-14 and LTR expression (Boehmer et al., 2004; Chelvarajan et al., 2005; Renshaw et al., 2002; Vega et al., 2004) or in the activation of molecules involved in LPS-linked signal transduction (such as p38 and JNK MAP kinases (Boehmer et al., 2004). Our studies indicate that age-related changes in macrophage activity are also a consequence of age-dependent changes in the extracellular milieu surrounding these cells in vivo. Important questions remain, including the possibility of reversing the effects by removal of the serum from aged rats, as well as the use of different combinations of cells and sera. It would be indeed very informative to investigate the effect of serum from adult and aged animals on cells from aged subjects, to see if the effects are additive or synergistic. Nevertheless, our studies clearly point out the need to consider the aged environment in molecular studies of the effect of aging on cell behavior. Of course, characterization of the factors responsible for each of the effects observed is of paramount importance, as their identification could lead to treatments designed to alleviate the effects of advanced age on the immune response. Our results also reinforce the idea that experimental conditions commonly employed in the literature, including the culture of cells in FBS, could result in masking of important age-related changes.

Acknowledgments

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