Original Article
OBESITY BIOLOGY AND INTEGRATED PHYSIOLOGY

Obesity

Immunosenescence Induced by Plasma from Individuals with Obesity Caused Cell Signaling Dysfunction and Inflammation

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Objective: To evaluate the consequences of plasma from individuals with obesity on parameters associated with immunosenescence in unrelated healthy peripheral blood mononuclear cells (PBMC).
Methods: Freshly isolated PBMC were incubated in media supplemented with 10% of plasma from individuals with obesity or control subjects for the first 4 hours of 24 to 120 hours of culture.
Results: Plasma from individuals with obesity modulated the phenotype of healthy PBMC, leading to a higher rate of apoptosis, lower amounts of phospho-γH2AX and -p53, and mitochondrial dysfunction. After 120 hours, there was a higher secretion of inflammatory cytokines IL-1β and IL-8. $CD8^+$ T lymphocytes presented decreased expression of CD28, which is associated with the immunosenescent phenotype. $CD14^+$ macrophages showed increased expression of CD80 and CD206, suggesting a modulation in the activation of macrophages.
Conclusions: These results demonstrate that chronic systemic inflammation observed in obesity induces dysfunctional features in PBMC that are consistent with premature immunosenescence.

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Introduction

Cellular senescence has been described as a state of permanent proliferative arrest of dividing cells (1). An increased proportion of senescent cells is detected in aged organisms or tissues and compromises tissue-repair capacity in the elderly (2,3). Fibroblast from older baboons also shows a time-dependent accumulation of DNA-damaged foci, another feature of senescent cells (4), and an increased presence of senescent cells has been reported in patients with chronic diseases such as diabetes (5) or atherosclerosis (6). In this regard, several lines of evidence suggest that senescence could have a causal effect on age-related tissue dysfunction, suggesting a common underlying mechanism between senescent cells, aging, and chronic diseases. Aging is also connected to a progressive impairment of the immune system. Age-associated functional decline of the immune compartment, increased production of inflammatory molecules, augmented release of oxidative stress mediators, and an imbalance toward increased circulating myeloid cells are observed during immunosenescence (7).

Senescent cells secrete large amounts of proinflammatory molecules and extracellular matrix proteases, called senescence-associated secretory phenotype (SASP). SASP can induce DNA, protein, and lipid damage in a paracrine way and might transform healthy cells, compromising cells' fate, tissue organization, and function (8), eventually leading to a systemic impairment of the organism. The molecular mechanisms associated with senescence induction in cellular response to genotoxic stress (DNA damage) involve the activation of a highly coordinated and regulated pathway called the DNA damage response (DDR). This pathway involves signaling of DNA

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damage through recruitment and phosphorylation of histone H2AX and the p53/p21 or p16 axis (2). Dysfunctional mitochondria also accumulate with age and can induce cellular senescence *in vitro* and *in vivo* (9,10).

Metabolic changes in white adipose tissue of individuals with obesity show increased infiltrating macrophages and senescent cells in fat tissue, contributing to oxidative stress and inflammation (11). Local deregulation is also reflected at a systemic level. Augmented circulating levels of inflammatory cytokines and pro-oxidant molecules are observed in the periphery contributing to the pathogenesis of obesity (11). In the past few years, many studies have demonstrated deleterious consequences of sustained inflammation and oxidative stress in obesity, such as DNA damage (12), telomere shortening (13), and premature senescence (14).

Because obesity and aging share similar features, such as reactive oxygen species (ROS) production and a wide array of pro-inflammatory cytokines associated with an impaired immune system, obesity is considered an aging disease associated with common mechanisms of premature immunosenescence (15). In this report, we incubated healthy peripheral blood mononuclear cells (PBMC) with plasma from individuals with obesity and analyzed the induction of the SASP in the long term. Our experimental model triggered compromised signaling of key features of the DDR, mitochondrial dysfunction, cytokine secretion, and immune deviation of T lymphocytes toward an immunosenescent phenotype.

Methods

Subjects

This study included 9 individuals with morbid obesity (BMI $\geq$ 35.0 kg/m^2) and 9 healthy controls (18.5 kg/m^2 $\geq$ BMI $\leq$ 24.9 kg/m^2). All participants with morbid obesity were recruited at the Center of Obesity and Metabolic Syndrome unit at São Lucas Hospital at Pontifícia Universidade Católica do Rio Grande do Sul. The Institutional Review Board from Pontifícia Universidade Católica do Rio Grande do Sul (N^o 760.537) and Universidade Federal do Rio Grande do Sul (N^o 640.817) approved this protocol. Eutrophic control individuals with no chronic inflammatory diseases were recruited from the community. A unique donor for each unrelated plasma sample was used as a donor of PBMC for cell culture experiments at different times ($n = 9$). All participants were older than 18 years and provided written informed consent.

Blood collection

Ten milliliters of peripheral blood were drawn from each subject by venipuncture into EDTA tubes. Blood was immediately centrifuged at 2,000 rpm for 10 minutes at 4°C for plasma separation. Collected plasma was aliquoted and stored at −80°C until needed.

Cell culture

PBMC were isolated by density gradient (Histopaque 1.077 g/mL; Sigma Aldrich, St. Louis, Missouri) according to manufacturer's instructions. Purified PBMC were counted manually with a hemocytometer and used if viability, assessed by exclusion of trypan blue, was greater than 95%. PBMC were cultured in 6 or 12 well plates at 1×10^6 cells/mL in RPMI-1640 medium (Sigma Aldrich) supplemented with 10% of plasma from individuals with obesity or healthy controls, 2 g/dL HEPES buffer (Sigma Aldrich), 1% penicillin/streptomycin (Sigma Aldrich), pH 7.4 in a humidified chamber incubated at 37°C with 5% CO_2 for 4 hours. For experiments, media was removed, cells were washed and replaced with RPMI media supplemented with 10% of fetal bovine serum (RPMI 10% FBS), and cultured for an additional 24 or 120 hours (Figure 1A).

Cell viability assays

Apoptosis and necrosis of cultured PBMC was analyzed with Annexin-V/PI (BD Biosciences, San Jose, California) staining according to manufacturer's instructions. Twenty-thousand events were acquired in an BD Accuri C6 flow cytometer (BD Biosciences) and analyzed with BD Accuri C6 software (BD Biosciences). Metabolic activity of total NAD(P)H-dependent cellular oxidoreductase enzymes was evaluated by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, Sigma Aldrich) as previously described (16).

Detection of intracellular phosphoproteins γH2AX and p53

Phosphorylation levels of γH2AX and p53 of cultured PBMC was evaluated by phosflow. The staining protocol consisted of an initial step of fixation and permeabilization by addiction of Perm/Fix Buffer (BD Biosciences) for 40 minutes at 4°C. Cells were then washed twice with Perm/Wash buffer (BD Biosciences) and resuspended in 100 μL of Perm/Wash buffer (BD Biosciences) with anti-phospho-H2AX (pS139, 1:20) conjugated to Alexa Fluor® 488 (BD Biosciences) and anti-phospho-p53 (pS37, 1:5) conjugated to Alexa Fluor® 647 (BD Biosciences) for 60 minutes at 4°C. Finally, cells were centrifuged and resuspended in Perm/Wash buffer. Acquisition (50,000 events) and analysis was done as reported.

Mitochondrial high-resolution respirometry

Mitochondrial physiology was measured by high-resolution respirometry in an oxygraph (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria). Cultured PBMC were placed in the 2-mL oxygraph chamber at a final concentration of 9×10^6 cells/mL of RPMI-1640 without serum as respiration media. Measurements were performed at a constant temperature of 37°C. Oxygen concentration and oxygen flux, which are directly proportional to oxygen consumption, were recorded with DatLab 4.3 (Oroboros Instruments). All experiments were performed at an oxygen concentration of 50 μM. First, basal oxygen consumption was recorded for 10 to 20 minutes. After stabilization of oxygen consumption, adenosine triphosphate (ATP)-synthase inhibitor oligomycin (1 μg/mL, Sigma Aldrich) was added to induce a state 4-like respiration independent of mitochondrial ATP production in which oxygen consumption is a consequence of proton leakage across the inner mitochondrial membrane. Maximal respiration, or oxygen consumption, was obtained by stepwise titration of the uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, Sigma Aldrich) until no additional increase in oxygen consumption was detected. Finally, the mitochondrial respiration was inhibited by adding potassium cyanide (KCN, Sigma Aldrich) in order to inhibit complex IV of the electron transport chain with no further decrease in oxygen consumption. The net residual oxygen flux reflects nonmitochondrial oxygen consumption and was subtracted from other respiration values. ATP-linked oxygen consumption was calculated as basal

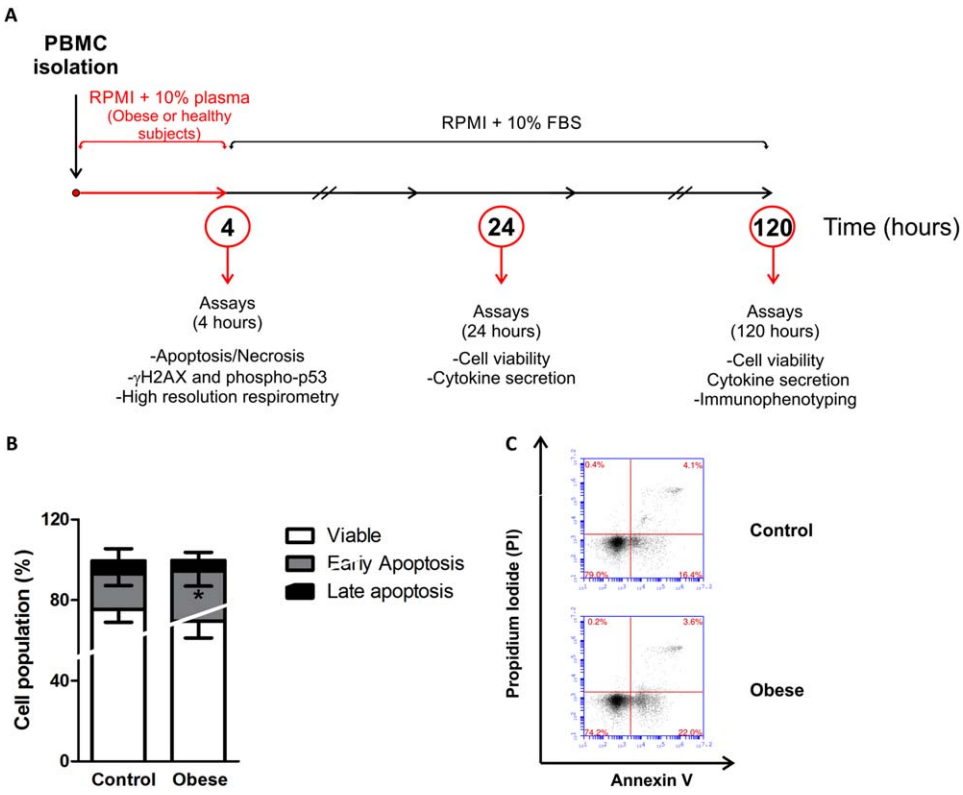

Figure 1 Increased apoptosis in PBMC exposed to plasma from individuals with obesity. (**A**) Workflow diagram. (**B**) Annexin-V and propidium iodide staining by flow cytometry. Plot showing significant increased early apoptosis in PBMC exposed to plasma from individuals with obesity after 4 hours of treatment. (**C**) Representative flow cytometry dot plot analysis in PBMC. Data presented as mean ± standard deviation. Significant differences considered when $P < 0.05$ (*). [Color figure can be viewed at wileyonlinelibrary.com]

respiration minus leak respiration. Reserve respiratory capacity was calculated as maximal respiratory capacity minus basal respiration.

Quantification of inflammatory cytokines

Cytokine secretion was measured in the culture medium of PBMC by Cytometric Bead Array with the Human Inflammatory Kit (BD Biosciences) according to the manufacturer's instructions. The samples were acquired in the Accuri C6 Flow Cytometer (BD Biosciences) and analyzed with the FCAP Array v3.0.1 software for BD CBA Analysis (Soft Flow Inc., Pecs, Hungary). Results are expressed as picograms per milliliter (pg/mL). The detection limits of the assays were 3.6 pg/mL for IL-8, 7.2 pg/mL for IL-1β, 2.5 pg/mL for IL-6, 3.3 pg/mL for IL-10, 3.7 pg/mL for TNF-α, and 1.9 pg/mL for IL-12.

Evaluation of senescent and inflammatory cell surface markers

Immunophenotyping was performed to analyze the expression of the lymphocyte senescent marker $CD28^+$ (anti-CD28 APC, 1:10) in $CD4^+$ and $CD8^+$ cells (Tritest anti-CD4 FITC, anti-CD8 PE, anti-CD3 PerCP, 1:10) and macrophage activation markers CD80 (anti-CD80 PE, 1:10), CD206 (anti-CD206 PE-Cy^{TM}5, 1:10), and HLA-DR (anti-HLA-DR APC, 1:10) in $CD14^+$ (anti-CD14 FITC, 1:10) cells after 120 hours of culture. Cells were stained with corresponding fluorochrome-labelled antibodies in staining buffer and incubated for 30 minutes at 4°C in the dark. Cells were then washed with staining buffer, and 20,000 events were acquired and analyzed in the monocyte or lymphocyte gates. All antibodies and reagents used were from BD Biosciences (San Jose, California).

Statistics

Statistical analysis was performed with the Statistical Package for Social Sciences (SPSS v.19 for Windows, IBM Corp,. Armonk, New York) and GraphPad Prism 6.01 (GraphPad Software Inc., La Jolla, California). The groups (eutrophic healthy controls vs. individuals with obesity) were compared using Student t test or Mann-Whitney U-test. All analyses were 2-tailed, and significance was set at $P < 0.05$. Data are presented as mean ± standard deviation.

Results

Demographic data is depicted in Table 1. There was no significant difference in age and sex. As expected, individuals with obesity had a higher BMI than healthy controls ($P < 0.0001$).

TABLE 1 Demographic and clinical data of healthy controls and individuals with obesity

	Healthy controls ($n = 9$)	Individuals with obesity ($n = 9$)	*P* value
Sex, male (%)	4 (44.4)	3 (33.3)	n.s.
Age, mean (SD)	29.8 (4.34)	35.8 (3.83)	n.s.
BMI, mean (SD)	21.3 (1.2)	50.3 (7.8)	< 0.0001
Comorbities (%)			
SH	–	6 (66.6)	
DM2	–	4 (44.4)	
MS	–	7 (77.7)	
DYS	–	7 (77.7)	
HS	–	3 (33.3)	

DM2, type 2 diabetes mellitus; DYS, dyslipidemia; HS, hepatic steatosis; MS, metabolic syndrome; n.s., not significant; SH, systemic hypertension.

Increased apoptosis in PBMC exposed to plasma from individuals with obesity

Plasma from patients with obesity is characterized by differential expression of circulating proteins and metabolites such as inflammatory cytokines, oxidative mediators, and adipokines that can be toxic to different tissues (17). To determine the impact of plasma from individuals with obesity on cell viability, we evaluated the percentage of cells in apoptosis and necrosis by Annexin-V and PI staining. We incubated PBMC from unrelated donor for 4 hours with RPMI supplemented with 10% plasma from individuals with obesity or from eutrophic healthy controls. A workflow diagram is depicted in Figure 1A. We observed an increased percentage of cells in early apoptosis in PBMC exposed to plasma from individuals with obesity (Figure 1B-1C). Similar results were obtained at 2 and 6 hours of incubation (data not shown).

Plasma from individuals with obesity compromises signaling through γH2AX and p53

Cell apoptosis is associated with DNA damage and has been documented in adipose tissue from humans and mice (18). However, the augmented apoptosis of circulating leukocytes is still under debate. In order to dissect consequences of the increased cell death in PBMC exposed to plasma from individuals with obesity, we assessed whether these healthy cells had higher DNA damage signaling through γH2AX and p53 phosphorylation. Surprisingly, PBMC exposed to plasma from individuals with obesity for 4 hours showed decreased γH2AX (Figure 2A-2B) and p53 (Figure 2C-2D) phosphorylation in viable cells.

Decreased routine and ATP-linked mitochondrial respiration in PBMC exposed to plasma of individuals with obesity

Loss of mitochondrial function can drive age-related decline in tissue function (10). Therefore, to further evaluate whether plasma from individuals with obesity was able to induce mitochondrial dysfunction, we performed a high-resolution respirometry assay. We observed that PBMCs acutely exposed to plasma from individuals with obesity for 4 hours showed reduced basal (routine) and ATP-linked oxygen consumption (Figure 3A-3B). We also evaluated cell metabolism and viability after 24 and 120 hours of PBMC culture by MTT (Figure 3C). We confirmed energy deficiency in PBMC exposed to plasma from patients with obesity after 24 hours ($P = 0.01$) and 120 hours ($P < 0.001$). Our results emphasize the noxious effect of plasma of individuals with obesity on immune cells.

Plasma from individuals with obesity modulates PBMC into an immunosenescent phenotype

As PBMC exposed to plasma from individuals with obesity showed signs of cell dysfunction, we wanted to know whether viable cells might become senescent after 120 hours of culture. We observed that plasma from individuals with obesity induced an increase in the percentage of immunosenescent TCD8+CD28- cells in healthy PBMC (Figure 4A-4C). Furthermore, CD4+CD28+ and CD8+CD28+ T cells showed a downregulation of the costimulatory molecule CD28 (Figure 4B-4C), suggesting the modulation of cytotoxic T lymphocytes into a senescent phenotype in the lymphoid compartment.

Increased cytokine secretion from PBMC exposed to plasma of individuals with obesity

Individuals with obesity present a low-grade inflammation. Indeed, in synergy with chronic activation of the immune system, this inflammatory status can be aggravated by the higher percentage of senescent cells that secrete bioactive molecules. In this regard, we evaluated whether plasma from individuals with obesity was capable of modulating the secretory inflammatory phenotype of PBMC after 24 and 120 hours of culture. After 120 hours of culture, we found that IL-1β and IL-8 (Figure 5) were significantly boosted following exposure to plasma from individuals with obesity for 4 hours, compared to healthy patients. IL-12 was not detected in any analyzed sample. Together with our previous data, these results support the deleterious effect of plasma from patients with obesity, suggesting that peripheral activation of immune system cells might indeed aggravate the SASP phenotype in obesity.

Increased macrophage activation markers in PBMC exposed to plasma of individuals with obesity

The degree of proinflammatory macrophage infiltration in white adipose tissue is associated with obesity (19). We asked whether the systemic inflammation observed in obesity can activate and differentiate monocytes into inflammatory macrophages after 120 hours of culture. While we did not observe significant differences in CD14 and HLA-DR expression markers, we detected increased expression of the costimulatory molecule CD80 and the mannose receptor CD206 in $CD14^+$ gated cells from PBMC exposed to plasma from individuals of obesity (Figure 6). Our *ex vivo* results reinforce a paracrine effect for the systemic activation of the immune system in obesity.

Discussion

Obesity is a multifactorial disorder that is strongly associated with accelerated aging and reduced life expectancy (15). In this study, we show that plasma from individuals with obesity induced increased

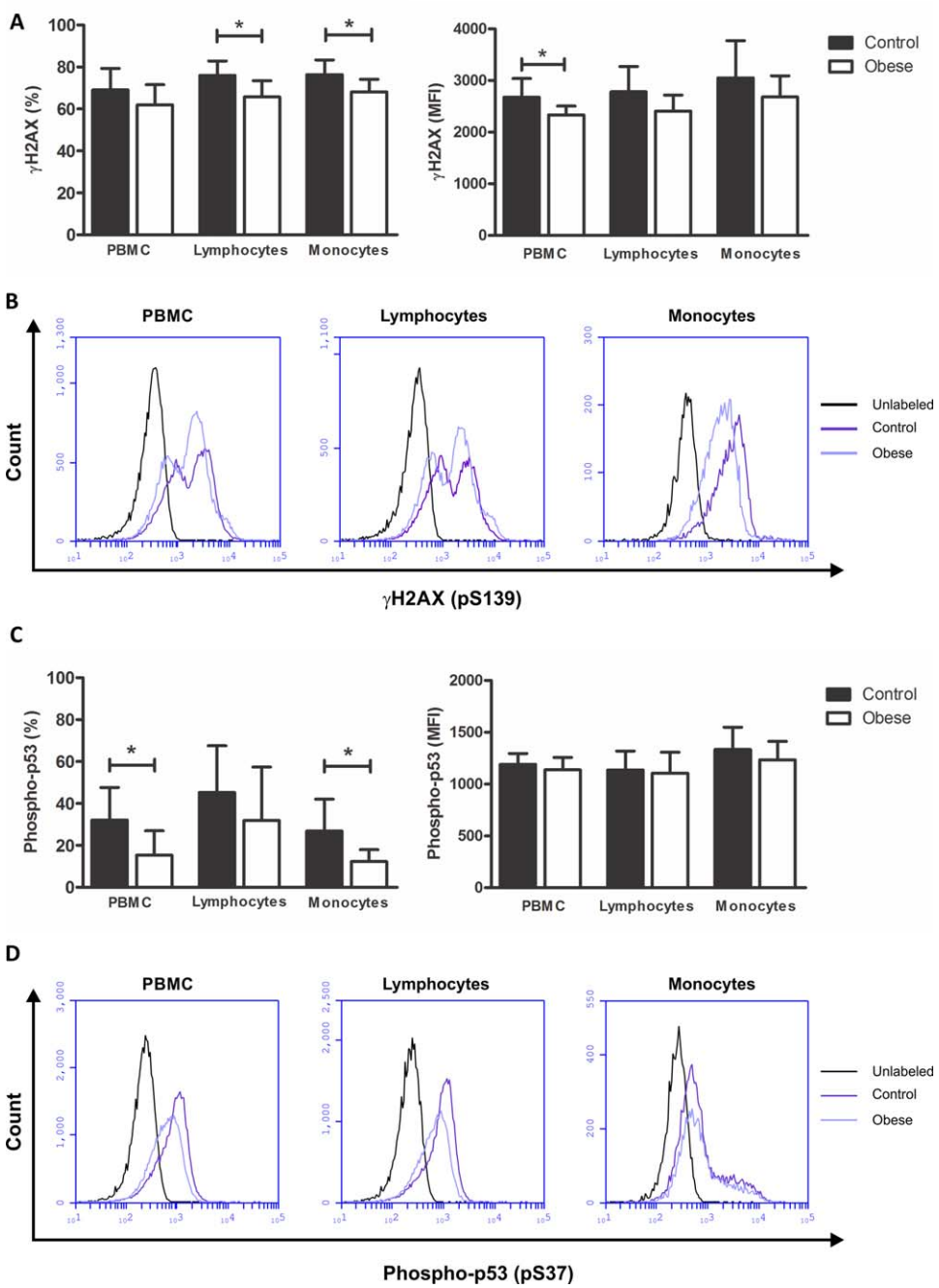

Figure 2 Plasma from individuals with obesity compromises signaling through γH2AX and p53. (**A**) Phosflow analysis showing significant reduction of H2AX phosphorylation (pS139) in PBMC and in gated lymphocytes and monocytes (based in FSC x SSC) exposed to plasma from individuals with obesity for 4 hours. (**B**) Representative histograms of H2AX phosphorylation (pS139) in PBMC and gated lymphocytes and monocytes (based in FSC x SSC) exposed to plasma from individuals with obesity for 4 hours. (**C**) Phosflow analysis showing significant reduction of p53 phosphorylation (pS37) in PBMC and in gated lymphocytes and monocytes (based in FSC x SSC) exposed to plasma from individuals with obesity for 4 hours. (**D**) Representative histograms of p53 phosphorylation (pS37) in PBMC and in gated lymphocytes and monocytes (based in FSC x SSC) exposed to plasma from individuals with obesity for 4 hours. Data presented as mean ± standard deviation. Student *t* test or Mann-Whitney U-test performed when appropriate. Significant differences considered when $P < 0.05$ (*). MFI: median fluorescence intensity. [Color figure can be viewed at wileyonlinelibrary.com]

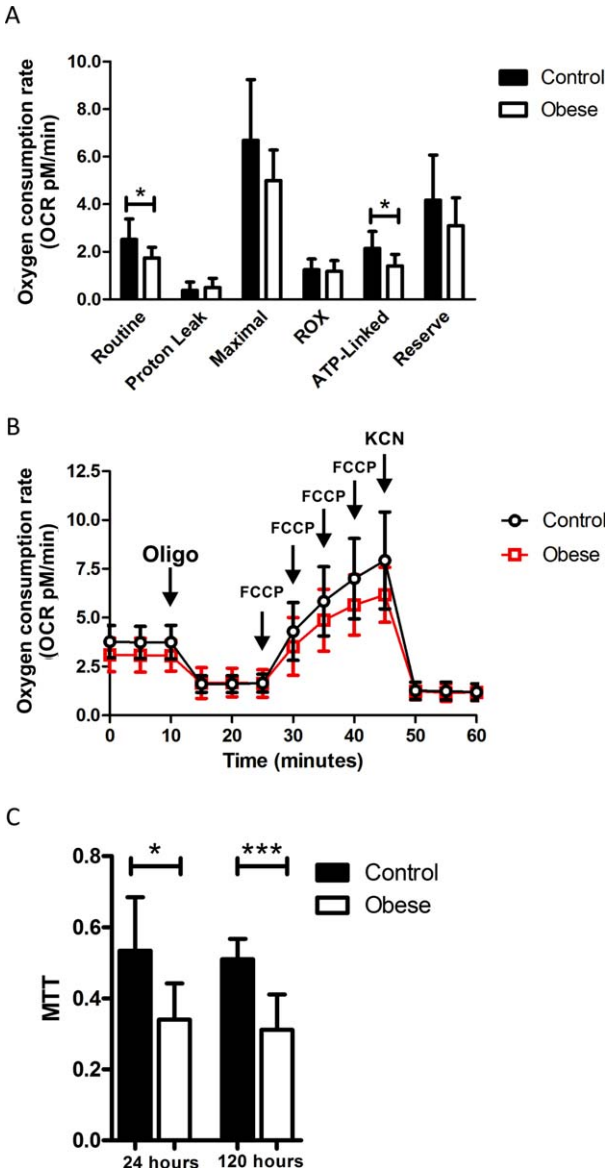

Figure 3 Routine and ATP-linked mitochondrial dysfunction in PBMC exposed to plasma of individuals with obesity. (**A**) Bar plot showing reduced routine and ATP-linked oxygen consumption rate (OCR) in PBMC exposed to plasma from individuals with obesity for 4 hours. Data are presented as mean ± standard deviation. (**B**) Representative curve of 3 points of OCR analyses in 4 stages of high-resolution respirometry assay (Oxygraph-2k). First, basal oxygen consumption was recorded for 10 to 20 minutes. After stabilization of oxygen consumption, ATP-synthase inhibitor oligomycin (oligo) was added to induce a state 4-like respiration independent of mitochondrial ATP production in which oxygen consumption is a consequence of proton leakage across the inner mitochondrial membrane. Maximal oxygen consumption was obtained by stepwise titration of the uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) until no additional increase in oxygen consumption was detected. Finally, the mitochondrial respiration was inhibited by adding potassium cyanide (KCN) in order to inhibit complex IV of the electron chain transporter, with no further decrease in oxygen consumption. The net residual oxygen flux reflects nonmitochondrial oxygen consumption and was subtracted from all respiration values. (**C**) MTT analysis. Plot showing reduced viability and energy deficit in PBMC cultured for 24 and 120 hours after 4 hours of exposure to plasma from individuals with obesity. *t* test or Mann-Whitney U-test performed when appropriate. Data presented as mean ± standard deviation. Significant differences considered when $P < 0.05$ (*) and when $P < 0.0001$ (***). [Color figure can be viewed at wileyonlinelibrary.com]

apoptosis of intact PBMC. In addition, viable PBMC showed reduced signaling through γH2AX and p53 pathways and mitochondrial dysfunction linked to reduced routine and ATP production. Plasma from patients with obesity modulated specific T lymphocytes into an immunosenescent phenotype associated with increased secretion of proinflammatory cytokines. Our findings support a causal dysfunctional response of PBMC triggered by the systemic inflammation in individuals with obesity.

Available data from proteome and genome-wide analyses arrays have identified particular genes or single nucleotide polymorphisms from the signaling glucose pathway, named insulin growth factor (IGF)-1 and IGF binding protein (IGFBP-3) present in plasma, associated with DM2, cancer, mortality (20), and age-related diseases (21). In addition, adipocytes secrete adipokines such as leptin and adiponectin, vascular endothelial growth factor, plasminogen activator inhibitor 1, macrophage migration inhibitory factor, monocyte chemotactic protein 1, C-reactive protein, and proinflammatory cytokines such as TNF-α, IL-1β, IL-8, IL-18, and IL-6 (15). We also have data regarding the enhanced pro-oxidant status of plasma from our cohort of patients with obesity (manuscript under preparation). All these factors contribute to the development of a systemic low-grade inflammation that has negative effects on tissues. In this regard, changes in one tissue might have a negative impact in several others (22). Successful experimental manipulations at the organ-specific (23) or systemic (22) level have proven to be promising and emphasize the systemic contagious aging effect.

As an initial step toward repair, cells respond to DNA damage by activating the DDR that can induce repair or trigger apoptosis or senescence via different signaling pathways (24). A common pathway involves recruitment and phosphorylation of histone H2AX at serine 139 (pS139). Although DDR foci detection by γH2AX phosphorylation might generally be accepted as a marker of DNA breaks, it should be noted that γH2AX presence should be interpreted as a readout of DDR and not as physical DNA damage. Therefore, a cell can experience DNA damage, even in the absence of detectable DDR foci, if upstream DDR signaling pathways are impaired (25). In this way, in the absence of γH2AX, initial recruitment of ATM might be unaffected, but the formation of DDR foci might be disrupted (25).

Proper signaling of γH2AX functions as an anticancer barrier and delays certain types of cancer by induction of cell death or senescence. Efficient DNA repair is essential for cell survival, and accumulation of defective repair machinery is an attribute of aging (26). Previous work has shown that PBMC from children with obesity were characterized by augmented γH2AX. However, in the context of inflammation, improper DNA repair was associated with formation of micronuclei, which might be regarded as a risk factor for cancer (12). In our settings, the harmful environment present on plasma from individuals with obesity blunted γH2AX signaling and impaired cellular resilience in lymphocytes and monocytes.

In agreement with reduced γH2AX signaling, p53 phosphorylation was also reduced. The requirement of p53 for cell cycle arrest induction is a well-established mechanism that acts in coordination with the cell cycle regulator p21 (27). The oxidative state present in adipose tissue is a trigger for DNA damage and, consequently, p53 activation. However, a recent study based on experimental evidence explored a simple network and suggested that p53 level of activation mediated by p38 MAPK can modulate cells' fate into apoptosis or

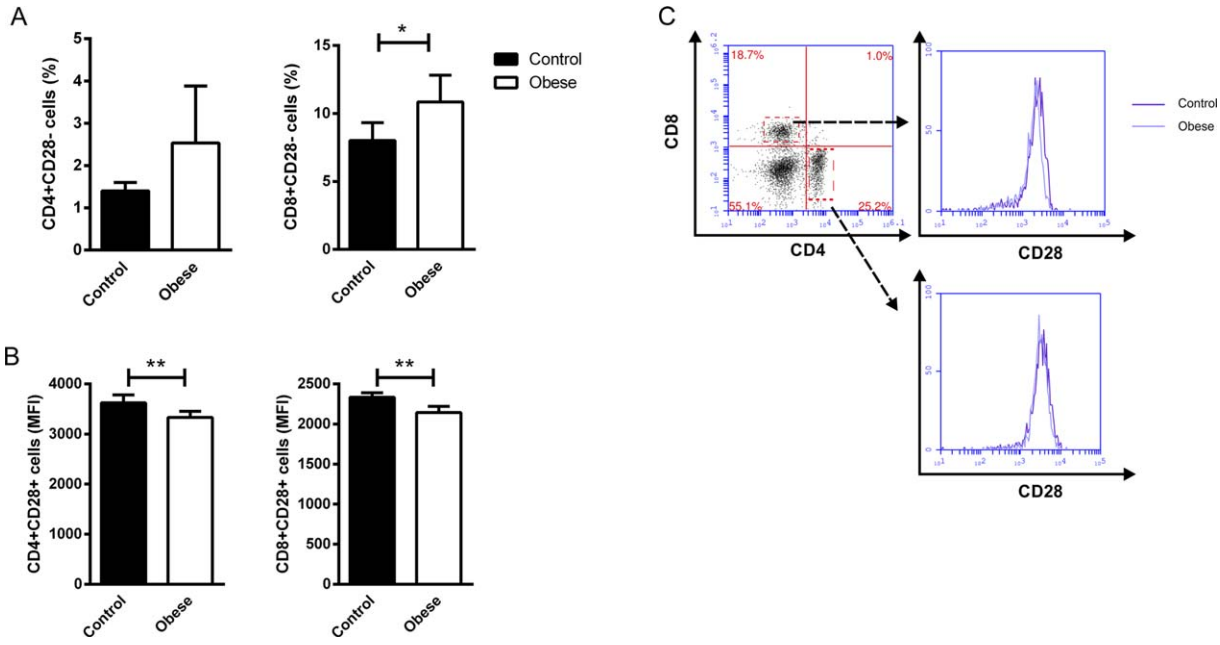

Figure 4 Immunosenescent phenotype of intact PBMC triggered by plasma from individuals with obesity. (**A**) Plots show CD8+CD28- immunosenescent cell population increase in PBMC cultured for 120 hours after 4 hours of exposure to plasma from individuals with obesity. (**B**) Plots show reduced expression of CD28 on surface of CD8+CD28+ and CD4+CD28+T lymphocytes in PBMC cultured for 120 hours after 4 hours of exposure to plasma from individuals with obesity. (**C**) Representative flow cytometry dot plots and histogram analyses in CD4 and CD8 lymphocytes. Data presented as mean ± standard deviation. Student *t* test performed, and significant differences were considered when $P < 0.05$ (*) and when $P < 0.01$ (**). MFI: median fluorescence intensity. [Color figure can be viewed at wileyonlinelibrary.com]

senescence through the upregulation of the cyclin-dependent kinase inhibitor $p16^{INK4a}$ (28). Although we did not measure $p16^{INK4a}$, we might speculate that the reduced p53 phosphorylation in our study could be a consequence of an early event of cellular senescence induced by the acute inflammatory stimuli. Another possible explanation for decreased p53 activation associated with augmented early apoptosis is a p53-independent mechanism (29).

In addition to apoptosis, after 5 days of culture, we observed an induction of a premature immunosenescent mechanism in T lymphocytes. Major changes on senescent $CD8^+$ and $CD4^+$ cells is the reduction of CD28 expression on the cell's surface (30). Corroborating with a senescent status, loss of CD28 is accompanied by shorter telomeres, decreased telomerase activity (31), and upregulation of cyclin-dependent kinase inhibitors $p21^{CIP1/WAF1}$ and $p16^{INK4a}$ (32). In this work, we were not able to measure universal hallmarks of cell senescence such as p21 and p16 expression and β-galactosidase activity. However, presence of signs of aberrant DNA damage response and SASP in PBMC exposed to plasma from individuals with obesity are also predictors of senescence induction. Immunosenescence affects functions of regulatory systems through an increased oxidative and inflammatory stress, resulting in age dysfunction and increased morbidity and mortality (11,15). Obesity is associated with an altered immune defense having a reduced response to pathogens and vaccines (33,34), while calorie restriction is able to decrease cell senescence and increase life expectancy and longevity (35).

Mitochondrial dysfunction has also been characterized as a hallmark of aging (36). Studies have demonstrated the importance of mitochondrial dysfunction and decreased ATP levels in senescence (9,10). Increased adenosine diphosphate/ATP ratios activate AMPK, which in turn can induce apoptosis or senescence through p53 or $p16^{INK4a}$ pathways (9). In addition, genetic manipulations that impair mitochondrial function without increase of ROS production accelerates aging, suggesting that dysfunctional mitochondria *per se* can contribute to aging independently of ROS (37). Furthermore, obesity is a pathological condition associated with reduced expression of mitochondrial DNA and decreased levels of proteins involved in oxidative phosphorylation in several tissues (15).

Plasma from individuals with obesity modulated PBMC into an inflammatory profile, as evidenced by macrophage activation and increased secretion of inflammatory cytokines. The adipokine leptin is increased in the circulation of individuals with obesity and is implicated in the regulation of monocyte and lymphocyte function (38), associated with production of TNF-α, IL-6, nitric oxide synthase 2, and ROS. NF-kB-driven inflammatory cytokines such as IL-6 and IL-8 contribute to a positive cycle that supports and stabilizes stress-induced senescence. Circulating proinflammatory cytokines are strong predictors of age-related morbidity and mortality (11). Furthermore, hypertrophied adipocytes release saturated fatty acids that serve as ligands for Toll-like receptor 4 (TLR4), thereby inducing inflammatory changes in macrophages through NF-kB activation (15).

At a cellular level, inflammation contributes to aging and decreases the regenerative potential of tissues. Proinflammatory signaling is integrated with stress and nutrient pathways such as p38 MAPK, TGF-β, and mTOR, which are involved in controlling mitochondrial

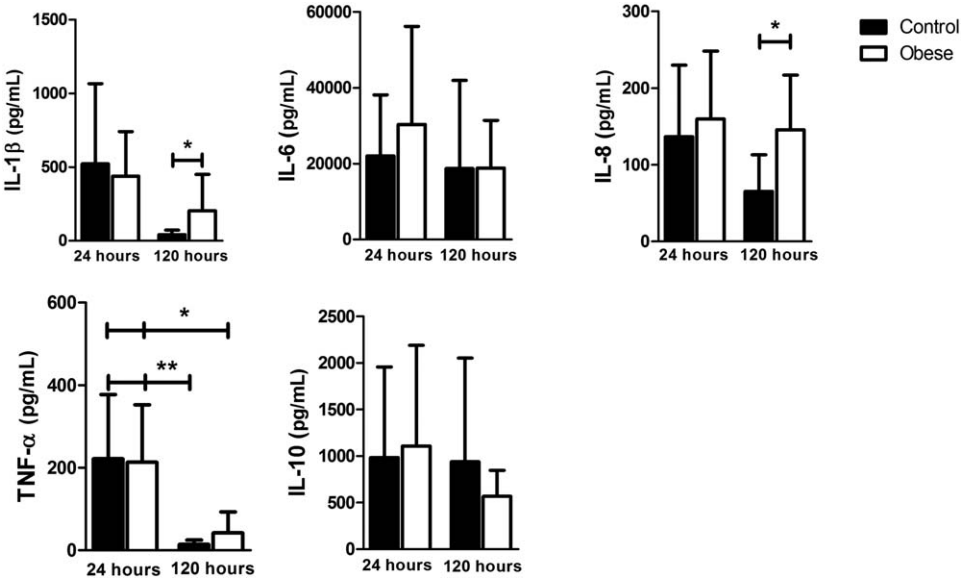

Figure 5 Enhanced cytokine secretion by PBMC exposed to plasma from individuals with obesity. (**A**) Plots show significant increase of IL-8 and IL-1β secretion by PBMC cultured for 120 hours after 4 hours of exposure to plasma from individuals with obesity. Data presented as mean ± standard deviation. Student *t* test or Mann-Whitney U-test performed when appropriate, and significant differences considered when $P < 0.05$ (*) and when $P < 0.01$ (**).

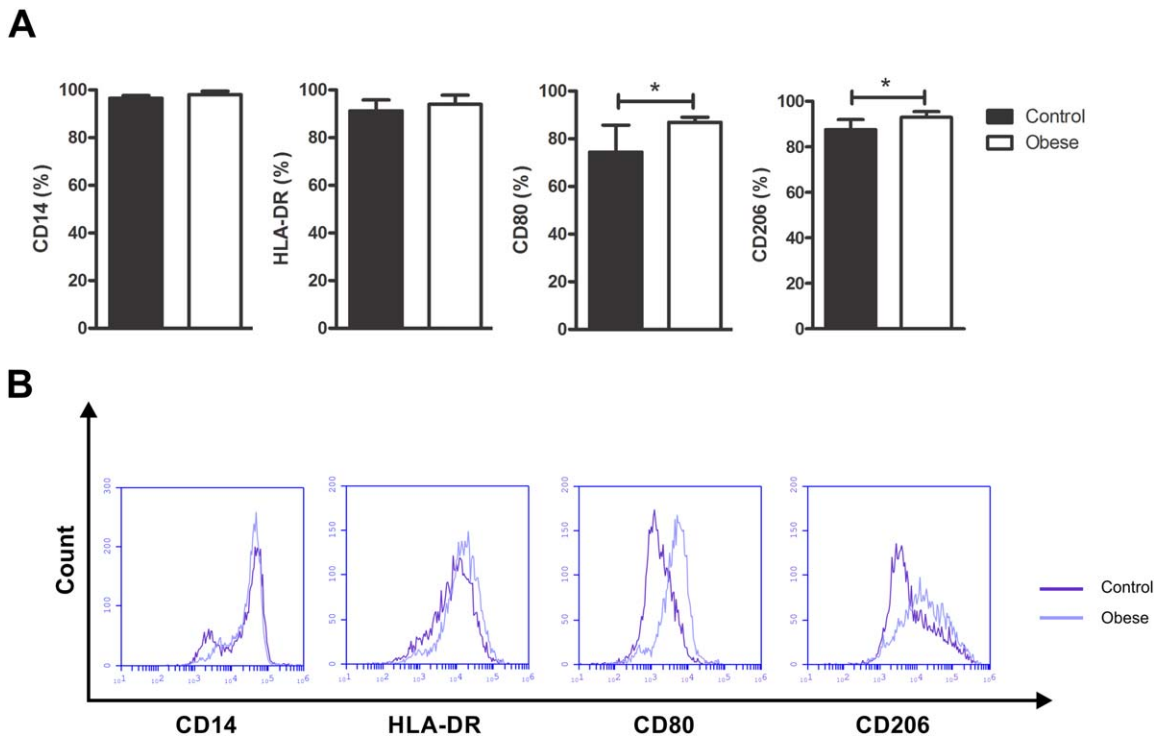

Figure 6 Monocyte to macrophage differentiation and subsequent activation triggered by acute stimuli from plasma from individuals with obesity. (**A**) Plots show significant increased surface expression of CD80 and CD206 markers in $CD14^+$ gated cells from PBMC cultured for 120 hours after initial 4-hour exposure to plasma from individuals with obesity. (**B**) Representative histograms of surface expression of CD14, HLA-DR, CD80, and CD206 markers in $CD14^+$ gated cells. Data presented as mean ± standard deviation. Student *t* test performed, and significant differences considered when $P < 0.05$ (*). [Color figure can be viewed at wileyonlinelibrary.com]

function and the production of free radicals (39). Thus, inflammatory mediators may be endogenous stressors of mitochondria and induce senescence and cell death (40). Premature accumulation of senescent cells enhances the oxidative and inflammatory signaling that induces DNA damage and mitochondrial dysfunction in a paracrine manner in surrounding cells in different tissues, creating a vicious cycle (8). In this context, our data support a causal role for chronic inflammation in obesity as a trigger of a pathological premature systemic aging phenotype.

A major limitation concerning our study was the reduced sample size. As expected, individuals with obesity presented important confounders such as comorbidities (systemic hypertension, type 2 diabetes mellitus, metabolic syndrome, dyslipidemia, and hepatic steatosis), all of them contributing to inflammation and oxidative imbalance. Also, valuable information regarding diet, latent or active viral infections, years of disease, and medication was missing and prevented us from exploring subgroup analyses to suggest further associations. Additional studies should be planned to determine the effect of plasma from healthy patients with obesity versus patients with obesity-related metabolic diseases.

Conclusion

Here we have presented data confirming the pathological consequences of plasma from patients with obesity. We have demonstrated a susceptibility for death and deterioration aggravated by the immunescent phenotype of lymphocytes induced by an acute stimulus. Our study emphasizes the need for further work exploring additional cellular and molecular mechanisms for the understanding of the aged phenotype observed in patients with obesity and aged-related diseases. O

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