Published in final edited form as:

Cell Immunol. 2020 February ; 348: 103994. doi:10.1016/j.cellimm.2019.103994.

Leptin induces immunosenescence in human B cells

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Abstract

Leptin is an adipokine secreted primarily by the adipocytes. Leptin has endocrine and immune functions and increases the secretion of pro-inflammatory cytokines by immune cells. Here we show that incubation of B cells from young lean individuals with leptin increases the frequencies of pro-inflammatory B cells and induces intrinsic B cell inflammation, characterized by mRNA expression of pro-inflammatory cytokines (TNF- α and IL-6), chemokines (IL-8), micro-RNAs (miR-155 and miR-16), TLR4 and p16, a cell cycle regulator associated with immunosenescence. We have previously shown that the expression of these pro-inflammatory markers in unstimulated B cells is negatively associated with the response of the same B cells after in vivo or in vitro stimulation. B cells from young lean individuals, after in vitro incubation with leptin, show reduced class switch and influenza vaccine-specific IgG production. Our results altogether show that leptin makes B cells from young lean individuals similar to those from young obese and elderly lean individuals, suggesting that leptin may be a mechanism of immunosenescence in human B cells.

Keywords

Leptin; Obesity; B cells; Immunosenescence

1. Introduction

Leptin is a pro-inflammatory adipokine secreted primarily by the adipocytes [1]. Similar to other pro-inflammatory mediators secreted by the adipocytes, leptin is released into the circulation. Plasma leptin levels are positively associated with body mass index (BMI). Leptin contributes to the pathogenesis of several autoimmune diseases [2] as well as to the development of inflammation in several non autoimmune conditions [3]. Leptin also influences several endocrine functions and regulates metabolism and energy homeostasis, suppressing energy intake and stimulating energy expenditure [4].

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Conflict of interest

The authors declare no conflict of interest.

Immune effects of leptin have been described [5]. Leptin acts on cells of innate and adaptive immunity. In general, leptin has pro-inflammatory functions and increases the secretion of pro-inflammatory cytokines by monocyte/macrophages [6–8], neutrophils [8], dendritic cells [9], NK cells [10, 11], Th1 [12] and Th17 [13] cells. These pro-inflammatory cytokines in turn up-regulate the secretion of leptin and acute phase proteins [14, 15], leading to the establishment of low-grade chronic inflammation. In B cells leptin increases intrinsic B cell inflammation, measured by phosphor-STAT3 in unstimulated B cells [16, 17], which is upstream of TNF- α , and it is therefore considered a negative regulator of B cell function, as B cell TNF- α is negatively associated with good B cell function [18].

Leptin secretion increases with aging [19–21] likely because the adipose tissue increases in size with aging [22, 23]. Increased leptin is at least one of the mechanisms which may be responsible for the reduced production of protective antibodies in elderly individuals and in individuals with obesity in response to vaccination [17, 24].

We have previously shown that aging is associated with poor B cell function, decreased production of protective antibodies and increased production of autoimmune antibodies. A reduction in activation-induced cytidine deaminase (AID), the enzyme necessary for class switch recombination, somatic hypermutation and IgG production [25, 26], as well as in its transcriptional regulator E47 [27], has been identified by our group and the molecular and cellular mechanisms involved have been thoroughly characterized [28–31]. Higher inflammation in serum, measured by TNF- α , has been shown to increase TNF- α production by B cells and this significantly decreases their capacity to make protective antibodies in response to antigenic/mitogenic stimulation [18].

In this study, we asked the question if leptin may be a mechanism of B cell aging. We compared phenotype and function of B cells from the following individuals: young lean, (Y_L) young obese (Y_O) and elderly lean (E_L) individuals, as well as of B cells from Y_L individuals in vitro treated with leptin. Our results herein show that leptin in vitro induces intrinsic B cell inflammation and pro-inflammatory B cells, reduced class switch and influenza vaccine-specific IgG production. B cells from Y_L individuals, after in vitro incubation with leptin, are similar to B cells from Y_O individuals, and also from E_L individuals. These results strongly support our hypothesis that leptin may be a mechanisms of immunosenescence in human B cells.

2. Methods

2.1. Subjects

Experiments were performed using blood isolated from Y_L (n=10 individuals, 25–55 years, BMI=21 kg/m²) and E_L (n=10 individuals, 65 years, BMI=23 kg/m²) individuals, as well as from the blood of Y_O (n=10 individuals, 25–55 years, BMI=36 kg/m²). The individuals participating in the study were screened for diseases known to alter the immune response or for consumption of medications that could alter the immune response. We excluded subjects with Diabetes, Congestive Heart Failure, Cardiovascular Disease, Chronic Renal Failure, malignancies, renal or hepatic diseases, autoimmune diseases, infectious disease, trauma or surgery, pregnancy, or documented current substance and/or alcohol abuse. Study

participants provided written informed consent. All individuals were vaccinated with the Trivalent Influenza Vaccine during the 2012–2013 and 2013–2014 Influenza vaccine seasons. The composition of the vaccines in the two seasons were: 2012–2013 (A/California/7/2009 (H1N1), A/Victoria/361/2011 (H3N2), B/Wisconsin/1/2010-like (Yamagata lineage)) and 2013–2014 (A/California/7/2009 (H1N1), A/Victoria/361/2011, B/Massachusetts/2/2012). All individuals were vaccinated each year since the 2009–2010 vaccine season. All experiments were performed using blood samples collected immediately before vaccination.

Study was reviewed and approved by the University of Miami Human Subject Research Office with Institutional Review Board IRB protocols #20070481 and #20160542, which reviews all human research conducted under the auspices of the University of Miami.

2.2. PBMC collection

PBMC were collected using Vacutainer CPT tubes (BD 362761) and cryopreserved. PBMC (1×10^6 /ml) were thawed and cultured in complete medium (c-RPMI, RPMI 1640, supplemented with 10% FCS, 10 μ g/ml Pen-Strep, 1mM Sodium Pyruvate, and 2×10^{-5} M 2-ME and 2 mM L-glutamine).

2.3. Flow cytometry

Cells at the concentration of 1×10^6 were stained for 20 min at room temperature with the following antibodies: anti-CD19 (BD 555415), anti-CD27 (BD 555441) and anti-IgD (BD 555778) to measure naive (IgD+CD27-), IgM memory (IgD+CD27+), switched memory (swIg, IgD-CD27+), and late memory (LM, IgD-CD27-) B cells. Up to 10^5 events in the lymphocyte gate were acquired on an LSR-Fortessa (BD) and analyzed using FlowJo 10.0.6 software. Single color controls were included in every experiment for compensation. Isotype controls were also used in every experiment to set up the gates.

2.4. B cell sorting and stimulation

B cells were sorted from the PBMC using magnetic CD19 Microbeads (Miltenyi), following manufacturer's instructions. Cell preparations were typically >98% pure.

For class switch and Ig production, B cells were stimulated at the concentration of 10^6 cells/ml with CpG (2 μ g/ml) for 1–8 days, to evaluate mRNA expression of E47 (at day 1) and AID (at day 5). For Ig secretion, culture supernatants were harvested at day 8.

To evaluate the effects of leptin on Ig class switch, B cells from Y_L individuals were stimulated in the presence of leptin (10 ng/ 10^6 B cells), alone or together with Rapamycin (RAPA, 10 μ M). We used human recombinant leptin (R&D Systems 398-LP-01M). RAPA was from Sigma Aldrich (53123-88-9).

To evaluate the effects of leptin on mRNA expression of pro-inflammatory markers, B cells from Y_L individuals were pre-incubated with leptin at the concentration of 10 ng/ 10^6 B cells for 60 min, then mRNA was extracted and qPCR performed.

To evaluate the effects of leptin on the induction of pro-inflammatory B cell subsets, B cells from Y_L individuals were pre-incubated with leptin at the concentration of 10 ng/10⁶ B cells for 24 hrs, stained with fluorochrome-conjugated antibodies and then analyzed by flow cytometry.

2.5. Cell proliferation

Cell proliferation was measured by ³H-Thymidine incorporation. Briefly, B cells at the concentration of 10⁶ cells/ml were left unstimulated or they were stimulated with CpG (2 µg/ml) for 2 days (optimal time point for cell proliferation, as determined in previous experiments). In the last 4 hrs of stimulation, cultures were pulsed with 1 µCi of ³H-Thymidine. Results are expressed as Stimulation Indices (S.I.): mean cpm from triplicate cultures in the presence of stimulus/mean cpm from triplicate cultures in the absence of stimulus.

2.6. mRNA extraction and quantitative (q)PCR

The mRNA was extracted from CpG-stimulated B cells at day 1 (E47) and at day 5 (AID), or from leptin-treated B cells, using the µMACS mRNA isolation kit (Miltenyi), according to the manufacturer's protocol, for quantitative (q)PCR. Reactions were conducted in MicroAmp 96-well plates and run in the ABI 7300 machine. Calculations were made with ABI software. Briefly, we determined the cycle number at which transcripts reached a significant threshold (Ct) for each target gene and for GAPDH as control. A value of the target gene, relative to GAPDH, was calculated and expressed as 2^{-Ct} . Reagents and primers (Taqman) were from Life Technologies.

2.7. ELISA to measure serum leptin and Ig in culture supernatants

Serum leptin was measured by Thermofisher kit BMS2039INST.

IgG and IgM in culture supernatants were measured by Bethyl kits E80–104 and E80–100, respectively, according to the manufacturer's instructions. Anti-influenza vaccine IgG were measured coating the ELISA plate with the same influenza vaccine used to vaccinate the individual. The vaccine was used at the concentration of 2 µg/ml.

2.8. Statistical analyses

Mean comparisons were performed by two-way ANOVA and by Student's t test (two-tailed), using GraphPad Prism version 7 software, which was used to construct all graphs.

3. Results

3.1. Leptin in vitro induces class switch defects in B cells from Y_L individuals, similar to those observed in B cells from Y_O and E_L individuals

We measured in vitro class switch and Ig secretion in B cells from Y_L , Y_O and E_L individuals, as well as in B cells from Y_L individuals in vitro treated with leptin. We evaluated E47 and AID mRNA expression by qPCR, and IgG and IgM secretion by ELISA. E47 is a transcriptional regulator of AID, the enzyme of class switch recombination and somatic hypermutation and a marker of optimal B cell responses, as we have previously

shown [29, 32–34]. We choose a dose of leptin that corresponds to the dose that is physiological for Y_O and E_L individuals (5X higher than in Y_L individuals), as shown in Fig. 1.

Cell proliferation is the first step in class switch. Therefore, we first measured cell proliferation by 3H -Thymidine incorporation in cultures of B cells from Y_L , Y_O and E_L individuals, as well as in B cells from Y_L individuals in vitro treated with leptin. Results in Fig. 2 (A) show that proliferation is higher in B cells from Y_L as compared to B cells from Y_O and E_L individuals. Fig. 2 (A) also shows that leptin does not have any toxicity and does not limit B cell division.

Then, we measured E47 and AID mRNA expression, as well as IgG and IgM secretion, in cultures of B cells from the same individuals. Results in Fig. 2 show that B cells from Y_O individuals, similar to B cells from E_L individuals, express significantly lower amounts of mRNA for E47 (B) and AID (C) compared to B cells from Y_L individuals and also secrete significantly less IgG (D) in culture supernatants. In vitro incubation of B cells from Y_L individuals with leptin induced a significant reduction in E47, AID and IgG, similar to the levels observed in B cells from Y_O and E_L individuals. No effects of aging, obesity or leptin in vitro were observed for IgM secretion (E).

3.2. Leptin in vitro decreases influenza vaccine-specific IgG production in B cells from Y_L individuals, similar to the levels observed in B cells from Y_O and E_L individuals

We tested the specificity of the IgG released in culture supernatants and we tested anti-influenza vaccine antibody production, as all participants received the influenza vaccine. We cultured B cells from Y_L , Y_O and E_L individuals with CpG for 8 days, to allow the stimulation/expansion of IgG secreting B cells. We also cultured B cells from Y_L individuals in the presence of leptin. Then we collected the supernatants that were tested for influenza vaccine-specific IgG production by ELISA. Results in Fig. 3 show decreased influenza vaccine-specific IgG antibodies in cultured B cells from Y_O and E_L individuals, as well as in cultured leptin-treated B cells from Y_L individuals, as compared to those from Y_L individuals.

3.3. Leptin in vitro induces mRNA expression of inflammatory markers in B cells from Y_L individuals, at levels comparable to those observed in B cells from Y_O and E_L individuals

The results above can be explained by the finding that leptin induces a pro-inflammatory phenotype in B cells from Y_L individuals. Briefly, we measured by qPCR mRNA expression of pro-inflammatory cytokines (TNF- α , IL-6), chemokines (IL-8), micro-RNAs (miRs, miR-155, miR-16), markers of immune activation (TLR4) and of cell cycle regulators (p16) in B cells from Y_L , Y_O and E_L individuals, as well as in B cells from Y_L individuals in vitro pre-incubated with leptin. These are the markers that we found negatively associated with class switch in our previously published studies [17, 18, 35]. Results in Fig. 4 show that the levels of expression of all these inflammatory markers are higher in B cells from Y_O and E_L individuals as compared to B cells from Y_L individuals. Moreover, leptin induces mRNA expression of these markers in B cells from Y_L individuals, the levels being similar to those in B cells from Y_O and E_L individuals. These results are in agreement with our previously

published findings showing spontaneous phosphorylation of STAT3 in B cells from Y_L individuals after in vitro incubation with leptin [17]. STAT3 binds several NF- κ B factors inducing their translocation from the cytoplasm to the nucleus and their activation, leading to spontaneous TNF- α production, which in unstimulated B cells is negatively associated with class switch and antibody production [18].

3.4. Leptin in vitro induces the expansion of pro-inflammatory B cell subsets

Aging [36, 37] and obesity [17] induce increased frequencies of the subset of pro-inflammatory B cells, called Double Negative (DN), also called late memory or tissue-like memory B cells. Independently from the way these cells are called, they are CD19+CD27-IgD-. This is the most pro-inflammatory B cell subset, which has been reported to be increased in the blood of patients with autoimmune [38–42] and infectious diseases [43–45], suggesting that these cells may expand in vivo in the presence of autoantigens or pathogen-derived antigens, in the context of a favorable inflammatory microenvironment, leading to the production of autoimmune or protective antibodies, respectively. DN B cells are transcriptionally active and affect the microenvironment by secreting pro-inflammatory mediators which in turn sustain and propagate the inflammatory response [36].

We compared the frequencies of DN B cells in Y_L , Y_O and E_L individuals, and in B cells from Y_L individuals after pre-incubation with leptin for 24 hrs. Results in Fig. 5 from this cohort of individuals show a significant increase in the frequencies of DN B cells in Y_O and E_L individuals, as well as in leptin-treated B cells from Y_L individuals, as compared to those in B cells from Y_L individuals.

3.5. RAPA rescues class switch in B cells from Y_L individuals in vitro treated with leptin

We then wanted to evaluate the possibility to rescue the defect in class switch induced in B cells from Y_L individuals by leptin. We compared AID mRNA expression and IgG secretion in cultures of CpG-stimulated B cells from Y_L , in vitro untreated or treated with leptin, alone or together with RAPA. Results in Fig. 6 show that RAPA was able to significantly reduce the leptin-induced defect in AID mRNA expression (A) and IgG production (B).

4. Discussion

The results in this paper show for the first time that leptin decreases in vitro class switch and influenza vaccine-specific IgG production in culture supernatants. The effects of leptin are associated with the induction of intrinsic B cell inflammation, measured by mRNA expression of pro-inflammatory markers. The major point of this paper is to show that B cells from Y_L individuals, after in vitro incubation with leptin, are phenotypically and functionally comparable to B cells from Y_O and E_L individuals, strongly supporting our hypothesis that leptin may be a mechanism of immunosenescence in human B cells.

Leptin is an adipokine secreted by the adipocytes and is involved in the regulation of immune functions in addition to its endocrine functions [1]. Most of the published work on leptin as regulator of immune responses has been performed on monocyte/macrophages, NK cells, T cells. Not so much work has been done on B cells so far. Previously published results on B cells have shown that leptin stimulates TNF- α and IL-6 production in a dose-

and time-dependent manner in vitro and this occurs through phosphorylation of JAK2 and STAT3 [16]. No data on the effects of leptin on antibody production have been reported so far. In addition to TNF- α and IL-6, we found several other markers of inflammation up-regulated by leptin, such as inflammatory miRs, TLRs and cell cycle regulators. The effects of leptin on the induction of mRNA expression for TNF- α and IL-6, and eventually of TNF- α and IL-6 secretion, may be due to leptin effects on lipolysis which have been described so far only for adipocytes [46] but may also occur in immune cells. Leptin has indeed been shown to inhibit the accumulation of lipids in adipocytes by increasing the turnover of triglycerides and their hydrolysis, a process leading to the generation of free-fatty acids and glycerol, which stimulate TNF- α and IL-6 secretion.

The leptin-induced inflammatory markers above have been shown in our previous studies to be negatively associated with antibody responses [17, 18, 35]. Here we found that class switch and anti-influenza vaccine IgG production are decreased in leptin-treated B cells from Y_L individuals, as compared to untreated controls. The amounts of anti-influenza vaccine antibodies induced by leptin in cultures of B cells from Y_L individuals are comparable to those observed in B cells from Y_O and E_L individuals. These results strongly suggest that leptin may be a marker of immunosenescence in B cells, in agreement with the findings that leptin secretion increases with aging [19–21] due to the fact that the adipose tissue increases in size with aging [22, 23].

Our results are the first to show that leptin decreases the secretion of protective antibodies in human B cells. We do not currently know if leptin also favors the production of autoimmune antibodies in human B cells. In mice, it has been shown that pristane injection induces lupus and leptin-deficient (*ob/ob*) mice are protected from the development of the disease and from the production of serum anti-ssDNA, anti-dsDNA and anti-chromatin autoimmune antibodies as compared to controls [47]. At the cellular level, leptin promotes effector T cell function [12] and inhibits T regulatory cell function [48]. Moreover, leptin facilitates the presentation of self-antigens to effector T cells, enhancing the availability of self-antigens released by apoptotic cells which favors the expansion of antigen-reactive T cells [49].

Our results also show that it is possible to counteract the effects of leptin, at least in vitro. We tested the effects of RAPA, the inhibitor of the mammalian Target Of Rapamycin (mTOR), as leptin has been shown to induce mTOR activation in T cells [12, 48] and macrophages [50]. Clinical studies have clearly demonstrated that mTOR inhibition with RAD001, an analog of RAPA, significantly improves influenza vaccine responses in elderly individuals [51]. Here we show that RAPA can counteract the leptin-induced down-regulation of class switch in human B cells, at least in vitro. The mechanisms of RAPA in B cells are not known.

In conclusion, our results show that leptin down-regulates class switch and antibody production. One potential mechanism can be the expansion of pro-inflammatory B cell subsets and the induction of inflammatory markers which are negatively associated with antibody responses as we have previously shown. Another potential mechanisms can be the induction of the inflammatory miRs, miR-155 and miR-16, that are decreasing the mRNA stability of AID and E47, respectively, as we have previously shown, leading to decreased

secretion of IgG antibodies. Our results suggest that limiting or blocking leptin may lead to up-regulation of antibody responses.

Acknowledgements

This study was supported by NIH R56 AG32576 (DF/BBB) and R56 AG059719 (DF). We thank nurses and staff of the Family Medicine Department. We are deeply thankful to all individuals who have agreed to participate in this study.

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HIGHLIGHTS

1. Leptin in vitro induces intrinsic B cell inflammation and pro-inflammatory B cells
2. Leptin in vitro decreases IgG production
3. Leptin may be a mechanism of B cell aging

Fig. 1. Higher serum leptin in Y_O and E_L individuals as compared to Y_L individuals. Serum leptin was measured by ELISA. Mean comparisons between groups were performed by Student's t test (two-tailed). Results are from 10 individuals/group. **** $p < 0.0001$.

Fig. 2. Leptin in vitro induces class switch defects in B cells from Y_L individuals, similar to those observed in B cells from Y_O and E_L individuals.

B cells were isolated from the peripheral blood using CD19 microbeads and positive selection. Cells were stimulated with CpG for 1–8 days. Results in **A** show cell proliferation as measured by ^3H -Thymidine incorporation and are expressed as Stimulation Indices (S.I.): mean cpm from triplicate cultures in the presence of stimulus/mean cpm from triplicate cultures in the absence of stimulus. Results in **B–E** show qPCR values (2^{-C_t}) of E47 (**B**) and AID (**C**) mRNA expression normalized to GAPDH $\pm$ SE. IgG (**D**) and IgM (**E**) production were measured in culture supernatants by ELISA. Mean comparisons between groups were performed by two-way ANOVA. Results are from 10 individuals/group.

** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Fig. 3. Leptin in vitro decreases influenza vaccine-specific IgG production in B cells from Y_L individuals, similar to the levels observed in B cells from Y_O and E_L individuals. B cells were stimulated with CpG for 8 days. Influenza vaccine-specific IgG antibodies were measured in culture supernatants by ELISA. ELISA plates were coated with the same vaccine used to vaccinate the person. Mean comparisons between groups were performed by two-way ANOVA. Results are from 5 individuals/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Fig. 4. Leptin in vitro induces mRNA expression of inflammatory markers in B cells from Y_L individuals, at levels comparable to those observed in B cells from Y_O and E_L individuals. B cells were isolated from the peripheral blood as indicated in Fig. 2 and left unstimulated. Heatmap shows qPCR values (2^{-Ct}) of several pro-inflammatory markers, normalized to GAPDH. Results show average qPCR values from 6 individuals/group. Mean comparisons between groups were performed by two-way ANOVA.

Fig. 5. Leptin in vitro induces the expansion of pro-inflammatory B cell subsets.

PBMC from Y_L , Y_O and E_L individuals, as well as PBMC from Y_L individuals pre-incubated with leptin for 24 hrs, were stained with anti-CD19, anti-CD27, and anti-IgD to measure swIg, IgM memory, naïve, DN B cells. **Top.** Representative dot plots to evaluate the different subsets in one individual/group are shown. **Bottom.** Results from 9 individuals/group are shown. Mean comparisons between groups were performed by two-way ANOVA. **** $p < 0.0001$.

Fig. 6. RAPA rescues class switch in B cells from Y_L individuals in vitro treated with leptin. B Cells from Y_L individuals were stimulated with CpG for 5 days (to evaluate AID mRNA expression, **A**) or 8 days (to evaluate IgG secretion in culture supernatants, **B**). B cell cultures were also treated with leptin, alone or together with RAPA. Results are from 4 individuals/group. Mean comparisons between groups were performed by two-way ANOVA. * $p < 0.05$, ** $p < 0.01$.