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MARINA CHAVES DE OLIVEIRA

EFFECT OF DIETARY COMPONENTS ON ARTHRITIS AND BONE REMODELING

EFEITO DE COMPONENTES DIETÉTICOS SOBRE A ARTRITE E REMODELAMENTO ÓSSEO

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ABSTRACT

Inflammation is an immune response that aims to protect the body against antigens and maintain the state of homeostasis. Consequently, this response leads to mobilization of metabolic substrates to generate an adequate support for it. Thus, both the inflammatory response interferes in intermediary metabolism as the nutritional status of the individual interferes with the inflammatory response. The first aim of this thesis was to evaluate the impact of acute joint inflammation on metabolism. Moreover, it was also aimed to determine whether the consumption of diets that alter the adipose tissue mass influences the inflammatory response. Acute joint inflammation in the knee of mice led to metabolic alterations. The use of inhibitor of cytokine instead of neutrophils appears to be a better alternative to improve these metabolic changes. On the other hand, chronic metabolic alterations induced by different dietary compositions altered the inflammatory response in the knee after challenge with antigen-induced joint inflammation. Together, we demonstrated that acute inflammation alters the metabolism. However, animals with metabolic abnormalities present joint inflammation attenuated. The second aim of this thesis was to analyze the effect of bovine-milk extracellular vesicles (BMEVs) on chronic arthritis and bone remodeling. BMEVs were caracterized by nanoparticle presence sized <220nm containing mRNA, miRNA, and proteins. In experimental chronic arthritis, it was shown that BMEVs are interesting nano/microparticle-based therapeutic agents to treat arthritis since reduce pro-inflammatory mediators and also attenuate the symptoms of the disease, although their in vivo mode of action remains to be elucidated. Thus, based on evidences in literature and our results, we also hypothesized that BMEVs could be used as tool for therapy of patients with arthritis. On the other hand, milk consumption is highly associated with bone formation, however it is still a controversial issue. BMEVs could interfere in the bone homeostasis, which is an orchestrated process between osteoclasts, osteoblasts and osteocytes. The fact that osteoblast differentiation is accelerated into osteocytes by BMEVs appears to influence dramatically the bone matrix leading to woven bone formation, which is more brittle and more vulnerable for fracture if not replaced by lamellar bone. Also, there was an increase in the differentiation of small osteoclasts, their activity was impaired and appeared to be related to a reduction in acid secretion. It was showed that nanoparticles in milk are modulators of immunity and bone by regulating arthritis response, osteoblast and osteoclast differentiation. Therefore, we demonstrated how dietary components may influence states of health and disease and this is important to create future strategies of therapies related to arthritis and bone.

Keywords: dietary components, arthritis, adipose tissue, bone, osteoclast, osteoblast

RESUMO

A inflamação é uma resposta imunitária que visa a proteção do organismo contra antígenos e a manutenção do estado de homeostase. Por conseguinte, esta resposta leva à mobilização de substratos metabólicos para gerar suporte adequado para o mesmo. Dessa forma, tanto a resposta inflamatória interfere no metabolismo intermediário como o estado nutricional do indivíduo interfere na resposta inflamatória. O primeiro objetivo desta tese foi avaliar o impacto da inflamação articular aguda sobre o metabolismo. Além disso, objetivou-se verificar se o consumo de dietas que alteram a massa adiposa influencia a resposta inflamatória. A inflamação articular aguda no joelho de camundongos levou a alterações metabólicas. A utilização de inibidor de citocina, e não de neutrófilos, parece ser a melhor alternativa para melhorar tais alterações. Por outro lado, as alterações metabólicas crônicas induzidas por diferentes composições dietéticas alterou a resposta inflamatória no joelho após o desafio com o antígeno indutor de inflamação articular. Em conjunto, demonstramos que a inflamação aguda altera o metabolismo. Entretanto, animais com alterações metabólicas apresentam inflamação articular atenuada. O segundo objetivo da tese foi analisar o efeito de vesículas extracelulares do leite bovino (BMEVs) na artrite crônica e remodelação óssea. BMEVs foram caracterizadas pela presença de nanopartículas de tamanho <220nm contendo RNAm, miRNA e proteínas. Na artrite experimental crônica, foi mostrado que BMEVs são agentes terapêuticos interessantes à base de nano/micropartículas uma vez que reduzem mediadores pró-inflamatórios e também atenuam os sintomas da doença, apesar de sua forma de ação in vivo ainda permanecer a ser elucidada. Assim, com base em evidências na literatura e nos nossos resultados, hipotetizamos também que BMEVs poderiam ser utilizadas como ferramentas para a terapia de pacientes com artrite. Por outro lado, o consumo de leite é altamente associado a formação óssea, no entanto, ainda é um fato controverso. BMEVs poderiam interferir na homeostase óssea que é um processo orquestrado entre osteoclastos, osteoblastos e os osteócitos. O fato de que a diferenciação dos osteoblastos é acelerada em osteócitos por BMEVs parece influenciar dramaticamente a matriz óssea que conduz à formação de tecido ósseo imaturo, que é mais frágil e mais vulnerável a fraturas, se não substituído pelo osso lamelar. Além disso, houve aumento na diferenciação de osteoclastos pequenos, sua atividade foi comprometida e pareceu estar relacionada com a redução da secreção de ácido. Foi mostrado que as nanopartículas no leite são moduladoras da imunidade e osso por regulação na resposta da artrite, e diferenciação de osteoblastos e osteoclastos. Portanto, nós demonstramos como os componentes da dieta podem influenciar os estados de saúde e doença e isso é importante para criar estratégias futuras de terapias relacionadas com a artrite e osso.

Palavras-chave: componentes dietéticos, artrite, tecido adiposo, osso, osteoclasto, osteoblasto

LIST OF ILUSTRATIONS

LIST OF ABBREVIATIONS AND ACRONYM

ALP	–	Alkalin Phosphatase
BMEV	–	Bovine-derived extracellular vesicles
BMI	–	Body mass index
BMP	–	Bone morphogenetic protein
CAF	–	Cafeteria diet
CD20	–	B-lymphocyte antigen CD20
CLA	–	Conjugated Linoleic Acid
CXCR1/2	–	CXC chemokine receptor 1 and 2
DC-STAMP	–	Dendritic cell-specific transmembrane protein
FFA	–	Free fatty acids
FGF	–	Fibroblast growth factor
HC	–	High refined carbohydrate-containing diet
IGF	–	Insulin-like growth factor
IL-1	–	Interleukin-1
IL-6	–	Interleukin-6
IL-6R	–	Interleukin-6 receptor
mBSA	–	Methylated Bovine Serum Albumin
MCP-1	–	Monocyte chemoattractant protein-1
M-CSF	–	Macrophage colony-stimulating factor
miRNA	–	microRNA
MMP9	–	Matrix metallopeptidase 9
mRNA	–	Messenger Ribonucleic acid
NFATc1	–	Nuclear factor of activated T cells c1
NF-κB	–	Nuclear factor kappa B
OPG	–	Osteoprotegerin
PDGF	–	Platelet-derived growth factor
PPARγ2	–	Peroxisome proliferator-activated receptor gama 2
PTH	–	Parathyroid hormone
RA	–	Rheumatoid Arthritis

RANK	–	Receptor activator of nuclear factor kappa-B
RANKL	–	Receptor activator of nuclear factor kappa-B ligand
RNA	–	Ribonucleic acid
RORγT	–	RAR-related orphan receptor gamma t
Runx2	–	Runt-related transcription factor 2
TGF-β	–	Transforming growth factor beta
Th1	–	Type 1 helper
Th17	–	Type 17 helper
TNF	–	Tumor necrosis factor
TRAF-6	–	TNF receptor-associated factor-6
TRAP	–	Tartrate-resistant acid phosphatase

SUMMARY

Chapter 1

Introduction and aims of the thesis

Chapter 1: Introduction

Evolution of human diet and its implication

Early evolutionary pressures resulted in conservation of energy for fighting infection and wound healing, while maintaining vigilance against attack. In a traditional view, the inflammatory response occurred by exposure to pathogens, leading mortality by infection and reduced cases of chronic inflammatory disorders. In modern times, the concern is more about environmental and psychosocial stress and medical illnesses, leading to reduced cases of infection, but with high rates of inflammatory conditions and autoimmunity (Miller e Raison, 2016). As in inflammation, diet composition also changed in these two evolutionary periods. Indeed, at the paleo age diet was based on the consumption of vegetables, tart fruits, nuts, wild meats, eggs, coconut and olive oil. Nowadays, people consume more refined and processed foods, sugars, candy bars, sweet fruits, juices, grains, bread, beans, genetically modified organism (GMO) foods, extracted seed oils, as well as dairy products (Jew *et al.*, 2009). Because of this change in diet composition, there was an increase in the incidence of chronic diseases, mainly inflammatory, such as obesity, diabetes, cancer, cardiovascular, pulmonary and neurological diseases, arthritis, autoimmune diseases and metabolic disorders (W.H.O., 2003; Jew *et al.*, 2009).

Immune response and metabolism

Wound healing, inflammation and the immune response against infection and damage are all processes that increase energy expenditure of an organism. Thus, many nutritional sensors and pathogen recognition systems need to operate in a coordinated fashion to mount a proper defense. The metabolic and immune systems are basically required to trigger this coordination, in which hormones, cytokines, signaling proteins, transcription factors and bioactive lipids are all involved (Figure 1) (Wellen e Hotamisligil, 2005). In general, the inflammatory response requires metabolic support and energy redistribution, and particularly the mobilization of lipid stocks plays an important role in combating infection during the acute phase of the inflammatory response (Khovidhunkit *et al.*, 2004), leading to increased levels of lipid in the circulation. Thus, the inflammatory response favors the catabolic and suppresses anabolic pathways.

During the inflammatory process the catabolism of glucose is also required to provide energy (Yu *et al.*, 2003). However, its mobilization when exacerbated can affect the insulin signaling pathway, leading to insulin resistance. This condition is defined by a loss of response to this hormone in target organs such as adipose tissue, liver and muscle. Insulin regulates the glucose uptake and the concentration of free fatty acids (FFA) (Reaven, 2005; Guilherme *et al.*, 2008). In adipose tissue, this hormone diminishes lipolysis, thereby reducing the efflux of FFA from adipocytes. On the other hand, the increased expression of proinflammatory cytokines, such as TNF-α occurring in obesity and acute inflammation, contributes to the opposite effect. High concentration of circulating FFA is one of the major factors that can cause insulin resistance in both animals and humans (Kelley *et al.*, 1993; Boden, 1997). Consequently, dyslipidemia can also be associated with this state due to the altered mobilization and storage of lipids (Lewis *et al.*, 2002). Since the amount of body fat induces insulin resistance, mechanisms underlying adipose tissue interfering with insulin signaling pathway have been studied extensively (Fantuzzi, 2005; Bastard *et al.*, 2006; Das, 2007). However, even in a lean state other dysfunctions can interfere in the metabolism, such as arthritis (Svenson *et al.*, 1988; Van Halm *et al.*, 2007; Oliveira *et al.*, 2016), stress (Mccowen *et al.*, 2001; Ricart-Jane *et al.*, 2002; Kyrou *et al.*, 2006), and systemic inflammation (Kyrou *et al.*, 2006), leading to an increase in lipids and glucose in the circulation.

Adipose tissue associated to metabolic and inflammatory alterations

Nutrients, metabolic products obtained after digestion of food in the body, are important to maintenance, growth and energy fuels for human development and survival (Koletzko *et al.*, 1998). Additionally, dietary factors are able to modulate the adiposity and induce metabolic dysfunction (Ng *et al.*, 2010; Sampey *et al.*, 2011). Numerous studies have demonstrated the association of diets rich in lipids with the development of obesity in both humans and animals (Shepard *et al.*, 2001; Cameron-Smith *et al.*, 2003; Weisberg *et al.*, 2006; Buettner *et al.*, 2007; Lee *et al.*, 2011). Moreover, it is also known that increasing the carbohydrate intake may contribute to obesity and related diseases (Lumeng *et al.*, 2007; Ferreira *et al.*, 2011). Although there are studies that assess metabolic changes triggered by diets rich in carbohydrates or lipids, studies are lacking that assess the effect of these diets on

the inflammatory response following an acute inflammatory stimulus, like during acute arthritis.

Adipose tissue is the most representative organ for fat storage, being its expansion associated with chronic diseases such as obesity, insulin resistance, diabetes, among others. Before the 90s, it was only known that the function of this tissue was related to energy reserves, protection against mechanical shock and thermal insulator. However, currently it is known that adipose tissue is a metabolically active organ and producer of adipocytokines, which interact with the entire organism, since they have both endocrine and inflammatory functions (Greenberg e Obin, 2006; Balistreri *et al.*, 2010). In obesity, when the expansion of adipose tissue occurs, particularly the visceral, there is an infiltration of inflammatory cells such as macrophages, which increase the production of inflammatory mediators, among these cytokines and chemokines. Then, it is settled a state of chronic inflammation of low intensity that affects not only the adipose tissue but also the brain, heart, pancreas, liver and reproductive system (Wellen e Hotamisligil, 2003; Gregor e Hotamisligil, 2011).

As described above, adipocytes produce inflammatory mediators called adipocytokines. These mediators play an autocrine, paracrine and endocrine function by interfering with the metabolism of lipids and glucose, contributing to the regulation of inflammatory processes (Vona-Davis e Rose, 2007; Penesova *et al.*, 2011). Among them, adiponectin secreted by adipose tissue is described to have anti-inflammatory properties and to beneficially contribute to the insulin sensitivity (Kern *et al.*, 2003). Leptin is produced by white and brown adipose tissue, placenta, and osteoblasts, being associated with appetite control and fat volume, and also regulation of differentiation and proliferation of osteoblasts (Thomas *et al.*, 1999; Cornish *et al.*, 2002; Gordeladze *et al.*, 2002). The adipocytokines act in the regulation of energy homeostasis, including self-regulation of growth and development of adipocytes (Maachi *et al.*, 2004). In obesity, adipocytokines such as leptin and resistin levels are increased, while adiponectin levels are reduced. The alterations of these adipocytokines are related to fat mass and insulin resistance (Ballantyne *et al.*, 2005). These same adipocytokines are altered in lean patients with rheumatoid arthritis in the serum (Migita *et al.*, 2006; Otero *et al.*, 2006) and knee

(Frommer *et al.*, 2010; Olama *et al.*, 2012), and they are associated with disease progression (Alkady *et al.*, 2011). Although evidence implies a role of adipocytokines in autoimmune diseases, their precise function in the pathogenesis and progression of arthritis remains to be elucidated.

Despite the strong association between obesity and other metabolic comorbidities, some peripheral inflammatory diseases such as arthritis, also became the focus of concern in relation to obesity influence on them. Obesity is considered as a possible enhancer pain and complications in arthritic patients (Janke *et al.*, 2007; Blagojevic *et al.*, 2010). According to Symmons *et al*. (1997) there is an association between Body Mass Index (BMI) above ≥30 kg/m^2 and increased risk for developing rheumatoid arthritis in both men and women. However, despite not having unraveled the mechanism in their study, they suggest that the risk would be more associated with metabolic dysfunction than mechanical issues (Figure 1). Few studies have analyzed the effect of body overweight over the pain or the disease of rheumatoid arthritis. Likewise, it is not known whether the metabolic and inflammatory changes seeing during obese or lipodystrophic state, resulting in an extreme change in adipose tissue size, can interfere in the arthritis response. Therefore, it becomes interesting to clarify whether the modulation of adipose tissue volume alters the pathological and immune response seen in arthritis.

Rheumatoid arthritis and therapies

Rheumatoid arthritis (RA) is a systemic autoimmune disease of unknown etiology characterized by inflammation of the joints, and can lead to functional impairment of the affected individuals. RA is considered the most common form of arthritis, affecting 0.5% to 1% of adults, with a higher incidence in women than in men (Alamanos e Drosos, 2005). T lymphocytes can represent up to 50% of synovial membrane cells from a patient with RA. Once activated, they produce pro-inflammatory cytokines that mediates angiogenesis, synovial hyperplasia, and proliferation of osteoclasts (Kong *et al.*, 1999). Treatment of patients aims to prevent joint damage, improve the quality of life and decrease joint pain (Anderson *et al.*, 1985).

In the last 10 years innovative protein-based therapeutic agents, called biologics or biological drugs, are developed to target cytokines or their cognate receptors

specifically (Finckh e Gabay, 2008). Examples of these biologics are antibodies, soluble receptors and receptor antagonists that block specifically cytokines (IL-1, TNF) or receptors (IL-6R, CD20), involved in the immune process (Kukar *et al.*, 2009). Despite the success of biologics in treatment of RA (Yazici *et al.*, 2008), their use at long-term may result in side effects. Therefore, the use of alternative therapies can reduce the consumption of drugs and limit unwanted effects.

The use of complementary or alternative therapies for RA patients, such as acupuncture, chiropractic, use of supplements, especially diets, is common among patients (Rao *et al.*, 1999). In respect to diet, it is believed that specific dietary components can mitigate the severity of symptoms by acting less aggressively as drug treatment (Martin, 1998). Studies have shown that there is indeed a correlation between the consumption of certain types of food or dietary components and the disease development (Berbert *et al.*, 2005; Mccann, 2007).

As RA is considered an inflammatory disease. Then food or nutrients known for their anti-inflammatory effects are generally considered protective (Figure 1) (Basu *et al.*, 2006). However, there is still no consensus on which particular type of diet would be the most appropriate to help these patients. Regular consumption of sugar-sweetened soda, but not diet soda, was also associated with increased RA risk in women [3]. On the other hand, alcohol consumption, specifically wine, as well as physical activity, presented an inverse association with RA risk [2]. Although there are still controversial data about omega-3 intake and RA, some studies showed a positive correlation with fish consumption and reduction of RA risk in woman at long-term [4] or suppression of clinical symptoms in RA [5]. Therefore, these studies indicate that the food composition could influence the arthritis risk and, its inflammatory response.

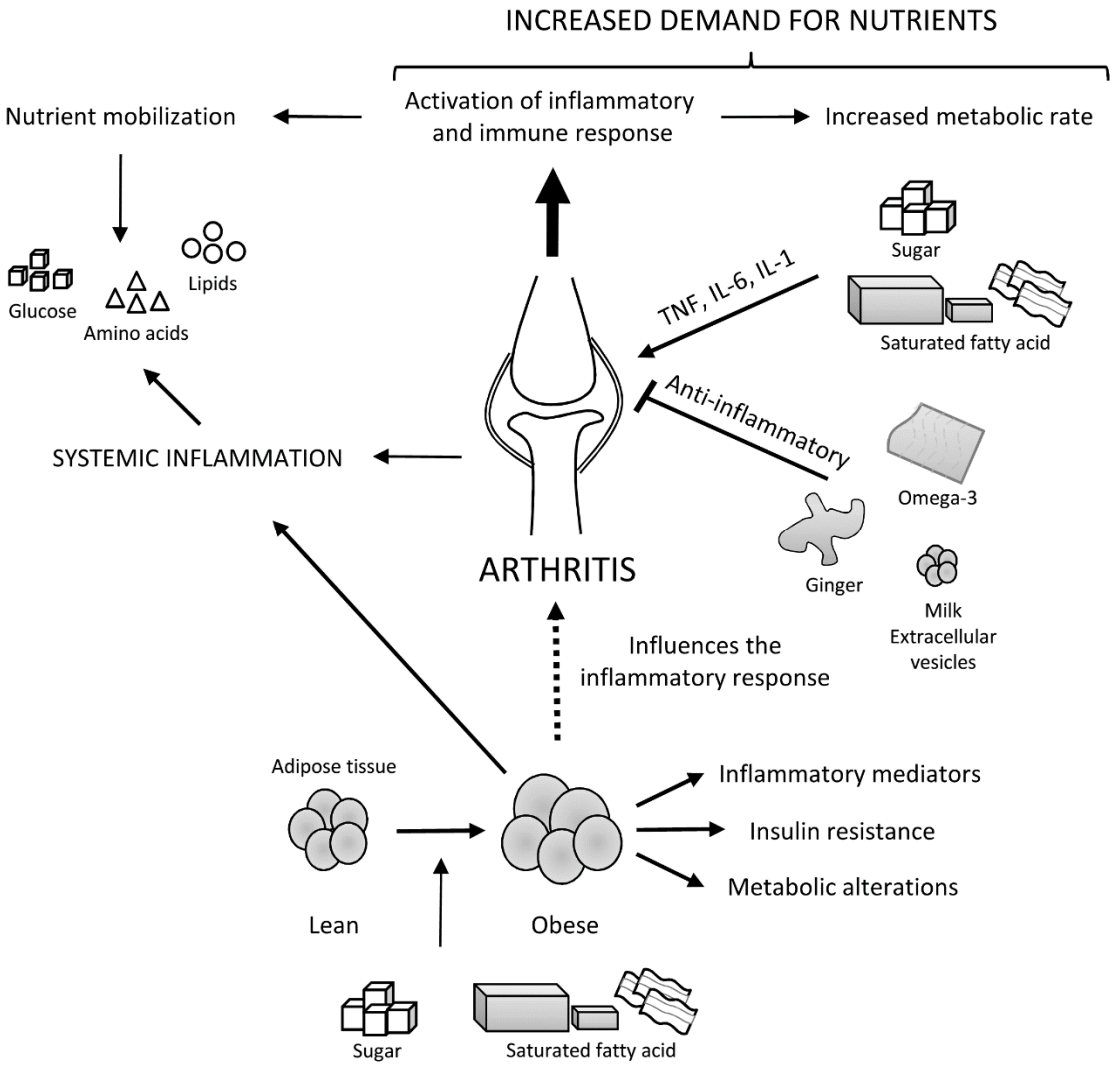

Figure 1 – The influence of food components on joint inflammation. Metabolic and immune systems are basically required to trigger the response against joint inflammation. These activation pathways increase the energy expenditure of an organism and leads to the mobilization of nutrients. Food components may disrupt or attenuate the inflammatory response, as well as the obese state may influence the joint inflammation. Therefore, hormones, cytokines, signaling proteins, transcription factors and bioactive lipids are all involved in the anti-inflammatory response. Dotted arrow: unknown effect.

Milk consumption and bone formation

Milk is considered a food with important nutritional value, highly consumed even after the breastfeeding period. It contains immunocompetent cells and soluble proteins that contribute to protection against infections (Armogida *et al.*, 2004). Another function of it, is to induce immune tolerance (Strobel, 2001) and promote the

protection against the development of allergies (Host *et al.*, 1999). Data indicate that economically developed countries generally consume more milk post-breastfeeding due to better living conditions and greater access to food (F.A.O., 2011). Bovine milk is mainly composed by the sugar lactose, high biological value proteins and fat with considerable concentrations of essential fatty acids (Jenness, 1979). Among the micronutrients, calcium is considered essential for the formation and maintenance of bones and teeth, as well as having an important role in muscle contraction, secretion of enzymes, cell division and aggregation (Ebashi e Endo, 1968; Kovacs, 2001).

Milk consumption is mainly associated with adequate bone formation. Studies in humans and mice have evaluated the effect of milk or its components on bone metabolism, showing an association with increased bone mineral density in women and children (Kalkwarf *et al.*, 2003; Uenishi *et al.*, 2007). Its consumption in childhood and adolescence provides greater bone density in postmenopausal (Sandler *et al.*, 1985). In mice, milk protein supplementation has the potential to improve the healing bone followed by fracture (Yoneme *et al.*, 2015). *In vitro*, the effect of milk components such as lactoferrin and whey protein (protein hydrolysate) on osteoblasts indicates increased cell proliferation and mineralization (Xu, 2009; Hou *et al.*, 2014).

On the other hand, other studies have shown an inverse relationship with respect to the consumption of milk and bone density. Recently, the consumption of milk more than three times a day was associated with the risk of fractures in both men and women, being referred to the presence of galactose in its composition (Michaelsson *et al.*, 2014). In postmenopausal women was observed a reduction of bone remodeling after six months of milk supplementation (Bonjour *et al.*, 2008). In a prospective study of 12 years it was evaluated whether the increased consumption of milk and / or other calcium rich foods reduces the incidence of osteoporotic fractures, but no association was found (Feskanich *et al.*, 1997). Another study showed that only the consumption of vitamin D was associated with lower risk of osteoporotic fractures, and this is not observed for milk and calcium in women after menopause (Feskanich *et al.*, 2003). Therefore, due these discrepancies concerning milk consumption on bone formation, it becomes difficult to establish a proper nutritional advice for healthy people and patients having bone diseases.

In recent decades, extracellular vesicles aroused the interest of researchers as inflammatory mediators and modulators of immunity (Vlassov *et al.*, 2012). Extracellular vesicles are nano membranous vesicles released by cells into the extracellular environment. Its origin can be endosomal or plasma membrane, called microvesicles or exosomes, respectively (Raposo e Stoorvogel, 2013). Studies have demonstrated that the extracellular vesicles contain proteins, RNA and microRNA (miRNA), and these vesicles stimulate target cells through the activation of surface receptors (Deregibus *et al.*, 2007) or gene transfer to other cells (Valadi *et al.*, 2007). Moreover, these molecules are highly stable and resistant to RNAse activity, pH and temperature extremes (Izumi *et al.*, 2012), indicating resistance to similar conditions present in the gastrointestinal tract. Recently, miRNAs present in milk were increased in the postprandial state in the serum of human and mice after its consumption (Baier *et al.*, 2014). These data indicate that these vesicles could be absorbed in the gastrointestinal tract and may have function elsewhere in the body. In the future, milk extracellular vesicles can be considered as a nutritional component that interferes in the homeostasis by regulating the metabolism and immunity.

Bone metabolism and cellular components

The skeletal structure consists of a hard connective tissue with the presence of specialized cells, mineralized and non-mineralized matrix, and spaces such as bone marrow cavity, vascular channels, tubules and gaps (Manolagas, 2000). Bone remodeling is a fundamental mechanism for maintaining the structural integrity of the skeleton as well as metabolic functions such as supply of calcium, phosphate and magnesium. Bone remodeling may be triggered by changes in the mechanical forces or micro-lesions and by hormonal responses, the latter to alter the supply of calcium and phosphorus. (Raisz, 1999). The physiological homeostasis of bone growth and skeletal remodeling is determined by the formation and resorption rate (Lerner, 2000). Changing local or systemic factors that activate bone remodeling and osteoclasts may trigger a turnover in bone mass (Lemaire *et al.*, 2004).

Bone homeostasis is an orchestrated process between osteoclasts, osteoblasts and osteocytes (Figure 2). The osteoclasts carry out bone resorption during the bone growth and remodeling, are derived from hematopoietic tissue and formed by the fusion of mononuclear cells (Lemaire *et al.*, 2004). Osteoblasts play an important role

in bone formation and are derived from mesenchymal stem (primitive) cells; these have the potential to auto-renew and can also lead to differentiation into fat cells, chondrocytes, hematopoietic cells, among others. The main function of osteoblasts is the formation of bone and subsequent matrix mineralization, in addition to regulating osteoclast bone resorption (Heino e Hentunen, 2008). Osteocytes are considered the last stage in the differentiation of osteoblasts and act as sensory cells for transporting information between osteoclasts and osteoblasts, contributing to the maintenance of bone matrix (Palumbo *et al.*, 2001); osteocytes are originated when osteoblasts are enclosed by the calcified matrix (Parfitt, 2002). Continuous production of new osteoclasts and osteoblasts, originated from their progenitors, is important to maintain bone maintenance (Manolagas, 2000). Besides regulation of bone formation performed by osteoclasts, osteoblasts and osteocytes, other cell types such as chondrocytes, and adipocytes, can also contribute to the homeostasis of bone marrow, since they have the same osteoblast progenitor, creating a competition between them.

Regulation of bone remodeling

The formation of osteoblasts is regulated by different signaling pathways (Figure 2). The transcription factor related to Runt (Runx2) and also osterix are considered critical for the differentiation of osteoblasts (Ducy *et al.*, 1997). The fact that Runx2 is also responsible for chondrocyte differentiation implies a common regulation of these two cells (Fujita *et al.*, 2004). The overexpression of Runx2 inhibits the maturation of osteoblasts, causing osteopenia and multiple fractures (Liu *et al.*, 2001). On the other hand, the peroxisome proliferator activated by gamma 2 (PPARy2) is an essential factor for differentiation of adipocytes and can inhibit the differentiation of osteoblasts via reduction of Runx2 (Liu *et al.*, 2010).

Osteoblasts produce various growth factors during the differentiation process, being their activity regulated in an autocrine and paracrine manner. The insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transformation growth factor-beta (TGF-β) and bone morphogenetic protein (BMP) act mainly on the regulation of Runx2 (Lieberman *et al.*, 2002). In addition to growth factors, osteoblasts produce extracellular matrix proteins to form the bone structure and ensure its quality through adequate deposition of collagen type I.

The osteocalcin and osteopontin are produced by osteoblasts and are indicative of the maturation phase of them, being osteocalcin produced in the post-proliferative phase, known as maturation phase, while osteopontin is present in the proliferative phase, or immature phase (Komori, 2010). Both operate in the modulation of mineralization, either by inhibiting through osteopontin or stimulating by osteocalcin (Hauschka *et al.*, 1989; Fujihara *et al.*, 2006). In addition to these proteins, alkaline phosphatase (ALP) is also an important enzyme in the process of mineralization, removing phosphate groups to be deposited in the bone matrix (Orimo, 2010).

On the other hand, the process of resorption is regulated by osteoclasts (Figure 2) that are formed when precursors of the monocyte-macrophage lineage fuse together to form multinucleated cells and become positive for Tartrate-resistant acid phosphatase (TRAP). TRAP is an intracellular enzyme responsible for degradation of internalized collagen in osteoclast vesicles, but being also used as a marker for the osteoclast differentiation process (Boyle *et al.*, 2003). The system osteoprotegerin (OPG) / RANKL / RANK regulates cell function by controlling osteoclastogenesis and bone remodeling. The RANKL acts on its receptor RANK, leading to the development of osteoclast differentiation, and can be stimulated and regulated through the presence of cytokines (Hofbauer *et al.*, 1999; Moonga *et al.*, 2002). In addition, RANKL expression is related to the state of osteoblast maturation, being increased in pre-osteoblasts (Atkins *et al.*, 2003). Besides that, OPG competes with RANKL by binding to RANK, acting as a repressor of osteoclast differentiation (Lacey *et al.*, 1998).

The monocyte-macrophage lineage is differentiated into mononuclear osteoclasts through the RANK / RANKL signaling, and activation of transcription factors TRAF6 and NFATc1. TRAF6 may activate different signaling pathways, such as NF-κB and MAPKs. Moreover, it induces the expression of c-Fos, which is an AP-1 family member (Takayanagi *et al.*, 2002; Kim *et al.*, 2010). Then, osteoclasts fuse via DC-STAMP and ATP6V0d2, forming multinucleated cells (Kim *et al.*, 2008). After differentiation, they can resorb bone using structures as a ruffled border and formation of actin ring, for isolating the area of resorption (Teitelbaum, 2000).

The irregular surface of osteoclasts presents proton pumps and chloride channels to promote acidification of the resorption lacunae (Coxon e Taylor, 2008). At the

beginning, the hydroxyapatite, which is considered as mineral matrix part, is dissolved and during this process the organic components are exposed and become susceptible of proteases action (Henriksen *et al.*, 2006). Among them, Cathepsin K and MMP9 are related to collagen type I degradation (Bossard *et al.*, 1996).

Besides regulation of signaling pathways, bone formation is also dependent on calciotropic hormones - parathyroid hormone (PTH), calcitonin and vitamin D (1,25-dihydroxyvitamin D) - responsible for regulating calcium metabolism and maintaining bone homeostasis (Gallagher *et al.*, 1998). The evidence presented above indicates that the bone metabolism has a regulation dependent of transcription and growth factors, cytokines, enzymes and hormones. A change in concentration and expression of these molecules may lead to either improvement or worsening of bone diseases.

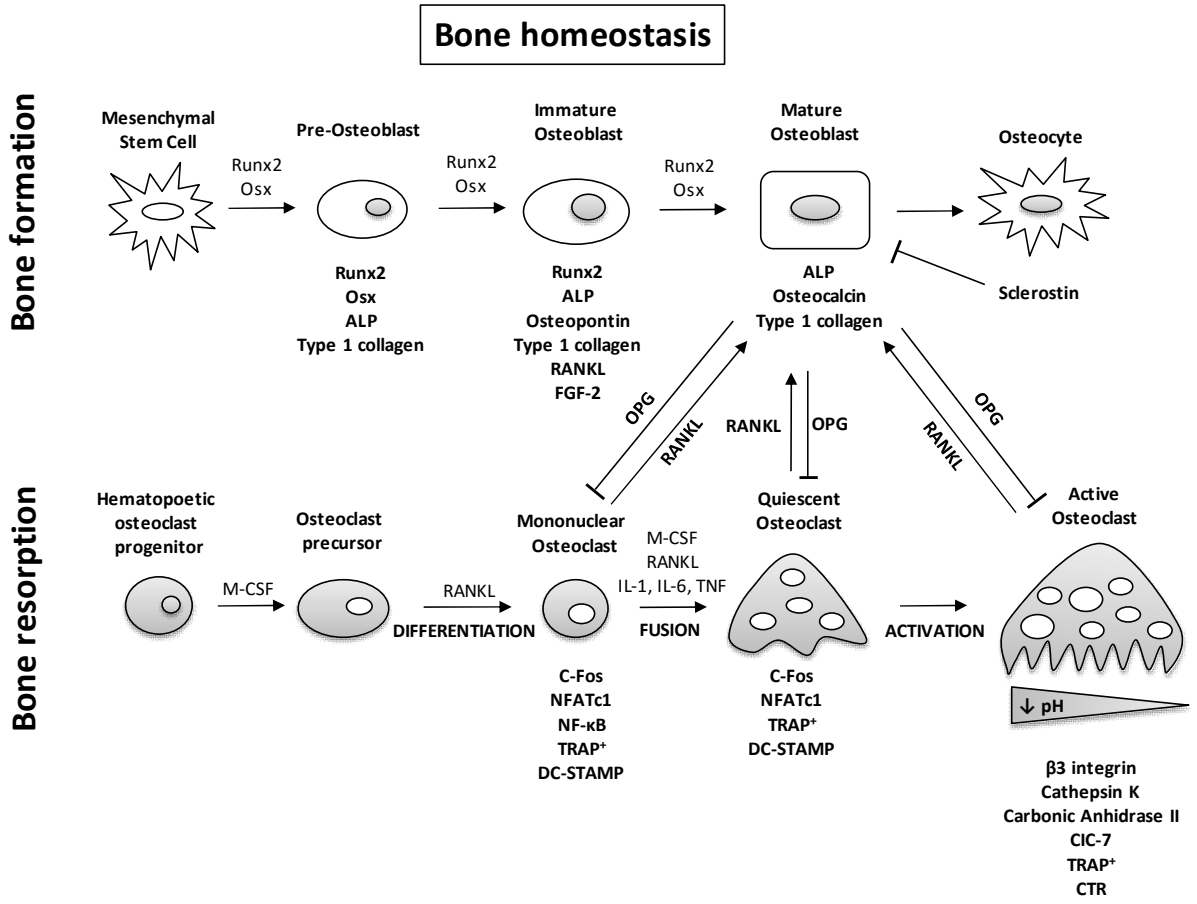

Figure 2 – Maturation process of osteoblasts and osteoclasts. Differentiation stages of precursor/stem cells into osteoblasts (osteoblastogenesis) and osteoclasts (osteoclastogenesis). Bone formation: Osteoblast differentiation starting from the mesenchymal stem cells and ending as osteocytes. Bone resorption: Differentiation phases of osteoclastogenesis from the hematopoietic stem cells, via pre-osteoclasts to functionally active, bone-resorbing osteoclasts. The osteoblasts influence the pre-osteoclasts to the osteoclasts through RANK/RANKL, an interaction that is blocked by OPG.

Aims and scope of the thesis

Nutrients are important determinants for health since they supply energy, building blocks and bioactive molecules that modulate key inflammatory and metabolic pathways. The general aim of this thesis was to study the interconnection between metabolism and inflammation on arthritis and bone response under a distinct dietary treatment.

The specific aims of this thesis were to investigate:

i) the impact of acute arthritis induced by antigen on metabolism. We also investigated whether the consumption of distinct diet composition interferes on the typical inflammatory response during the acute arthritis.

ii) the effect of bovine-milk extracellular vesicles on chronic arthritis and bone remodeling response.

Frequently, inflammatory diseases can be associated with metabolic alterations that may also, as a feedback, contribute to the improvement or worsening of the disease. Considering the first specific aim, in **Chapter 2**, we analyzed the consequences of gonarthritis induced by knee injection of the antigen mBSA on metabolism. For that, we evaluated alterations in the metabolism of glucose and lipids, as well as adipocytokines production using an acute model of antigen-induced arthritis in mice. Furthermore, we treated mice with Etanercept, an anti-TNF drug, DF2156A, a CXCR1/2 receptor antagonist, or a monoclonal antibody RB6-8C5 to deplete neutrophils, in order to determine the mechanism that caused the metabolic alterations.

Adipose tissue is one of the main organs associated with low grade inflammation and metabolic dysfunction seen during the obesity. Consumption of diets, generally rich in lipids or refined carbohydrates, can alter the adipose tissue mass and induce distinct metabolic alterations. In **Chapter 3,** we verified how the remodeling of the adipose tissue and associated metabolic dysfunction could influence the acute joint inflammatory response induced by antigen in mice. Then, we used again the model of antigen-induced joint inflammation to evaluate the inflammation in the knee and

peri-articular tissue, as well as, the reaction of infrapatellar adipocytes and production of adipocytokines.

Chronic arthritis is also described to be influenced by the intake of dietary components. Milk is widely consumed all over the world even after the breastfeeding phase. We studied one of its components, the extracellular vesicles, on inflammation. Extracellular vesicles were detected in milk as small particles composed basically of mRNA and microRNA, and may have immunoregulatory properties. We explored in **Chapter 4** the consequences of bovine-milk extracellular vesicles (BMEVs) intake on disease response in two mouse models of arthritis, collagen-induced arthritis (CIA) and the IL-1Ra gene knockout mouse strain.

Based on previous results about the effect of BMEVs on chronic arthritis, we described in **Chapter 5** a hypothesis that the consumption of BMEVs may also influence positively the arthritis disease treatment through modulation of inflammatory response in human therapy.

Milk consumption or its components are highly related to bone metabolism. In the last two chapters (**Chapter 6 and 7**), we aimed to investigate the effect of BMEVs on bone remodeling. In **Chapter 6**, we analyzed the influence of BMEVs on bone matrix formation *in vivo* after intake of BMEVs in the drinking water by mice. In addition, we also evaluated the osteoblast differentiation *in vitro*, verifying alterations in mineralization, proliferation and collagen deposition, as well as, genes related to it.

Next, in **Chapter 7**, we complemented the effect of BMEVs on bone, by also analyzing its influence on osteoclast differentiation *in vitro* and *in vivo*. To do this, bone-marrow cells of mice were differentiated into osteoclasts and the process of differentiation and their activity were studied. Also, mice received orally BMEVs in the drinking water.

Finally, we showed in **Chapter 8** a general discussion and a summary of the results of this thesis and established potential applications and perspectives on future research.

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Chapter 2

Tumor necrosis factor, but not neutrophils, alters the metabolic profile in acute experimental arthritis

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RESEARCH ARTICLE

Tumor Necrosis Factor, but Not Neutrophils, Alters the Metabolic Profile in Acute Experimental Arthritis

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Abstract

Metabolic alterations are associated with arthritis apart from obesity. However, it is still unclear which is the underlying process behind these metabolic changes. Here, we investigate the role of tumor necrosis factor (TNF) in this process in an acute model of antigen-induced arthritis (AIA). Immunized male BALB/c mice received an intra-articular injection of PBS (control) or methylated bovine serum albumin (mBSA) into their knees, and were also pre-treated with different drugs: Etanercept, an anti-TNF drug, DF2156A, a CXCR1/2 receptor antagonist, or a monoclonal antibody RB6-8C5 to deplete neutrophils. Local challenge with mBSA evoked an acute neutrophil influx into the knee joint, and enhanced the joint nociception, along with a transient systemic metabolic alteration (higher levels of glucose and lipids, and altered adipocytokines). Pre-treatment with the conventional biological Etanercept, an inhibitor of TNF action, ameliorated the nociception and the acute joint inflammation dominated by neutrophils, and markedly improved many of the altered systemic metabolites (glucose and lipids), adipocytokines and PTX3. However, the lessening of metabolic changes was not due to diminished accumulation of neutrophils in the joint by Etanercept. Reduction of neutrophil recruitment by pre-treating AIA mice with DF2156A, or even the depletion of these cells by using RB6-8C5 reduced all of the inflammatory parameters and hypernociception developed after AIA challenge, but could not prevent the metabolic changes. Therefore, the induction of joint inflammation provoked acute metabolic alterations which were involved with TNF. We suggest that the role of TNF in arthritis-associated metabolic changes is not due to local neutrophils, which are the major cells present in this model, but rather due to cytokines.

Introduction

Systemic metabolic alterations are not only caused by obesity and their associated comorbidities, but also linked to autoimmune diseases such as arthritis [1]. Arthritis is characterized by an infiltration of inflammatory cells, cartilage and bone destruction, and it is clinically presented as pain, swelling and stiffness of affected joints [2]. Inflammatory cytokines and chemokines play a pivotal role in the local and systemic inflammation of arthritic patients, contributing to the disease development and progression [3]. Despite it is not well explored, neutrophils also participate in arthritis progression, and evidences indicate that neutrophil influx does occur during recurrence of disease [4]. Moreover, lean patients diagnosed with arthritis have shown alterations in serum levels of adipocytokines, which are released mainly from the adipose tissue and are also associated with arthritis progression [5].

Different categories of drugs are routinely used for the treatment of arthritis, aiming to relieve symptoms and avoid progression of the disease [6]. Although some components of the arthritic inflammatory response still need to be unveiled, there have been significant developments in the last decades, including novel immunobiological agents targeting tumor necrosis factor (TNF). In addition to providing relief to patients, these agents have been shown to improve metabolic alterations associated with arthritis [7, 8]. However, mechanisms describing the relationship among soluble mediators and systemic metabolic alterations still need to be elucidated.

We have previously described a local production of TNF-α and chemokine (C-X-C motif) receptor 2 (CXCR2)-mediated neutrophil influx following antigen challenge in a model of antigen-induced arthritis (AIA) in mice [9, 10]. Here, we report that there is also a systemic metabolic alteration after acute induction of AIA. We then investigated the relative contribution of TNF for the observed systemic metabolic changes, and which is the relation to its known inflammatory role.

Materials and Methods

Ethical Approval

All experiments with mice were approved by the "Ethics Committee in Animal Experimentation at Universidade Federal de Minas Gerais" in Brazil (protocol: 148/2012).

Animals

Eight-week-old male BALB/c mice were obtained from the animal care center at Universidade Federal de Minas Gerais. It was also used mice with a lysozyme M promoter for enhanced green fluorescent protein (LysM-eGFP), expressing fluorescently neutrophils for the confocal microscopy analysis. They were maintained in an environmentally controlled room under a 12/12 h light-dark cycle, with filtered water and food *ad libitum*. During the procedures for arthritis induction, mice were anesthetized with 1.5% isoflurane in oxygen. After the indicated time points, mice were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) and killed. Samples of the blood, knee, epididymal adipose tissue and liver were collected for further analysis.

Arthritis induction and assessment of articular inflammation

Mice were immunized i.d. at the base of the tail with 500 μg of methylated BSA (mBSA) in 100 μL of an emulsion containing saline and an equal volume of complete Freund's adjuvant. The knee challenge was performed 14 days later. For the control group, each mouse received an intra-articular injection in both knee joints with 10 μL PBS. This group is represented by the

mean of all time points evaluated. For AIA mice, they were injected with 10 μg mBSA in 10 μL PBS [9]. After the antigen challenge, mice were killed at the indicated time points. The knee cavity was washed with PBS (2x 5 μL) for cell recovery. The total number of leukocytes was determined by counting leukocytes in the synovial fluid by manual counting using Neubauer chamber under optical microscopy after staining with Turk's solution. Differential counts were obtained from cytospin preparations (Shandon III, Thermo Shandon, Frankfurt, Germany) stained with May—Grünwald—Giemsa. The peri-articular tissue was removed for determination of myeloperoxidase activity and cytokine/chemokine measurement by ELISA.

Drugs

Etanercept (Enbrel®) was diluted in saline and administered i.p. (10 mg/kg) in a volume of 100 μL 30 min before the arthritis induction. The allosteric CXCR1/2 inhibitor DF2156A (10 mg/kg) was diluted in carboxymethylcellulose 0.5% and given orally per gavage 30 min before arthritis challenge (Biogen-Dompé, Italy). Anti-GR-1 MAb (RB6-8C5 clone) was injected i.v. (1 mg/mL) in a volume of 100 μL twice, one day and 1 hour before arthritis induction (eBioscience, San Diego, CA). Control PBS and AIA groups received the same vehicle and administration route of the drug correspondent in each experiment.

Nociception assessment

In a quiet room, the mice were placed in acrylic cages (12x10x17 cm high) with a wire grid floor, 15–30 min before the test, for environmental adaptation. A series of stimuli was performed only when the animals were quiet, without exploratory movements or defecation. In these experiments, an electronic pressure meter was used. It consists of a hand-held force transducer fitted with a polypropylene tip (INSIGHT Instruments, Ribeirão Preto, São Paulo, Brazil) [11]. A non-standard large tip (4.15 mm^2) was adapted to the probe [12]. An increasing perpendicular force was applied to the central area of the plantar surface of the hind paw to induce the flexion of the knee joint, followed by paw withdrawal. A tilted mirror below the grid provided a clear view of the animal's hind paw. The end point was characterized by the removal of the paw from the polypropylene tip. After the flexion-elicited withdrawal threshold, the intensity of the pressure was automatically recorded. The value for the response was obtained by averaging two measurements in one hind paw.

Total and differential blood cell counts

Blood was collected from the tail vein of mice and the total white blood cells were counted using a Neubauer chamber. Peripheral blood smears were stained with May-Grünwald-Giemsa, and the differential white blood cell count was determined under oil immersion (1000x).

Histology

The knee joint was removed and fixed for 24 h with 4% a neutral-buffered formalin. Then, the joints were incubated in 14% EDTA at pH 7.2 during 4 weeks at room temperature for decalcification. The samples were embedded in paraffin and sections of 7 mm were stained with Hematoxilin-eosin. Two sections/knee joint were microscopically examined and scored in a blind manner for different parameters [13], as follows: severity of synovial hyperplasia (ranging from 0 to 3) and intensity and extension of inflammatory infiltrate (ranging from 0 to 4). The grades were summed to obtain an arthritis index (ranging from 0 to 7).

Confocal Microscopy

Immunized LysM-eGFP mice (eGFP-expressing neutrophils) were challenge with a knee injection of PBS or mBSA and after 3 and 24 hours the confocal microscopy was performed as described before [14]. Briefly, mice were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg). Epididymal adipose tissue and liver were exposed over a Plexiglas support, and the tissue microcirculation was visualized under confocal microscopy using an Olympus Fluoview FV300 laser-scanning microscope equipped with a 488 nm argon laser. All images were acquired using a 10x magnification objective lens, and three different fields from each mouse were analyzed.

Oral glucose tolerance test (OGTT)

After 24 hours of PBS or mBSA knee injection, mice were fasted overnight and received D-glucose (2 mg/g body weight) orally per gavage. Levels of glucose were measured from the blood collected from the tip of the tail at 0, 15, 30, 60, and 90 min after D-glucose administration using a glucometer (Accu-Check, Roche Diagnostics, Indianapolis, IN).

Metabolic analyses in the serum

Total cholesterol, triglyceride and glucose levels were quantified by enzymatic kits (KATAL, Belo Horizonte, MG, Brazil). Adiponectin, resistin and leptin levels were assayed by ELISA (R&D systems Europe Ltd., Abington, UK) as well as insulin (Millipore, Bedford, MA). The insulin resistance index was calculated as follows: HOMA-IR = fasting glucose level (mmol/L) x fasting insulin level (μU/mL) ÷ 22.5.

ELISA assay

Samples of peri-articular tissue, epididymal adipose tissue and liver were homogenized with a protease inhibitor solution (1:10). This solution contained 0.4 M NaCl, 0.05% Tween 20, 0.5% bovine serum albumin, 0.1 mM fluoride fenilmetilsufonila, 0.1 mM benzethonium chloride, 10 mM EDTA, 20 IU of aprotinin diluted in a solution of phosphate buffer (8g NaCl, 0.2g KCl and 2.89 g $Na_2HPO_4.12H_2O$ diluted in 1 liter of distilled water). The homogenate was centrifuged at 10,000 rpm for 10 minutes at 4°C and the supernatant of knee and liver, and the infranatant of adipose tissue were collected to measure cytokine/chemokine levels by ELISA. Levels of chemokine (C-X-C motif) ligand 1 (CXCL1) were determined in the peri-articular tissue, epididymal adipose tissue and liver, and pentraxin 3 (PTX3) was evaluated in the serum using DuoSet ELISA development kits (R&D System, Inc., Minneapolis, MN, USA), in which cytokines are detected using a sandwich antibody system according to the manufacturer's instructions.

Myeloperoxidase (MPO) activity

Indirect neutrophil presence was measured by assaying MPO activity, as described previously [15]. Briefly, peri-articular tissue, epididymal adipose tissue and liver samples of mice were homogenized and assayed for MPO activity by measuring the change in OD at 450 nm using tetramethylbenzidine.

Leukocyte analysis by flow cytometry

After blood collection (0.1 ml), the red blood cells were lysed with ACK buffer. Subsequently, cells were incubated with the specific monoclonal antibody for markers of the main cellular populations present in the peripheral blood, GR1 and CD11b (neutrophils), CD3 (T cells) and

F4/80 (monocytes) (BD Pharmingen). Cells were analyzed with a FACSCalibur CantoII, and data were analyzed by FlowJo (TreeStar).

Statistical analysis

Data are expressed as mean ± SEM. Multiple comparisons were performed using one-way ANOVA: it was used the Dunnett's post-hoc test in the kinetic results; for experiments with drugs the Newman-Keuls post-hoc analysis and; in the OGTT two-way ANOVA followed by the Bonferroni post-hoc test. The statistical analysis was performed using the software GraphPad Prism (GraphPad Software, La Jolla, CA). The statistical significance was set at $P<0.05$.

Results

Acute arthritis induces accumulation of neutrophils in the adipose tissue and liver, and triggers metabolic alterations

At 24 and 48 hours after AIA induction, mice had a significant hypernociception, which is an index of pain, as assessed by the lower paw withdrawal threshold compared with control mice (Fig 1A). This acute phase of AIA was also characterized by an increased recruitment of neutrophils into the knee joint and peri-articular tissue (MPO activity), which peaks at 24 hours after challenge (Fig 1B and 1C). Neutrophil accumulation in the synovial fluid resolved at 48 hours, but it was still present at this time in the peri-articular tissue. The recruitment of neutrophils was mirrored by the presence of the neutrophil-active chemokine CXCL1 in the peri-articular tissue (Fig 1D). Histopathological analysis showed an intense infiltration of neutrophils and increased synovial hyperplasia (Fig 1E and 1F). There was also an increase in MPO activity in the epididymal adipose tissue and liver, suggesting a significant influx of neutrophils in these two organs (Fig 1G). Despite this increase in neutrophil infiltration, we did not observe alterations in the levels of CXCL1 chemokine in the adipose tissue, but only in the liver at 1, 3 and 48 hours after AIA challenge (Fig 1H). Using Lysm-eGFP mice, which present eGFP-expressing neutrophils, we showed an increase in the neutrophil accumulation in the adipose tissue at 24 hours and in the liver at 3 and 24 hours following arthritis induction (Fig 1I and 1J), concurring with the MPO data.

In order to verify whether the metabolism of mice was affected when subjected to AIA, we evaluated the major systemic metabolic parameters after antigen administration in the joint of immunized mice. AIA mice showed alterations in the glucose and lipid metabolism, as seen by an acute hyperglycemia, insulin resistance, glucose intolerance, hypertriglyceridemia and increased cholesterol levels (Fig 2A–2E). The adipocytokines, which are released mainly by adipose tissue, were also altered in AIA mice. Levels of adiponectin reduced while levels of leptin increased in specific time points (Fig 2F and 2G). There were no changes in resistin levels (data not shown). Altogether, these results demonstrate that mice challenged with a local antigen-specific inflammatory stimulus had significant systemic metabolic changes.

The metabolic changes in AIA mice are dependent of TNF

The blockade of TNF may prevent the inflammatory response and hypernociception in this model of AIA [10]. To evaluate the relevance of TNF in the systemic metabolic changes observed after induction of AIA, mice were treated with Etanercept, a known TNF inhibitor in humans and mice [16, 17]. The pre-treatment with Etanercept reduced all the inflammatory parameters developed after AIA challenge (Fig 3A–3E). Interestingly, glucose and lipid parameters were also improved (Fig 3F–3I), and serum levels of adiponectin and leptin were normalized in mice treated with Etanercept (Fig 3J). PTX3 is a serum reactive protein rapidly

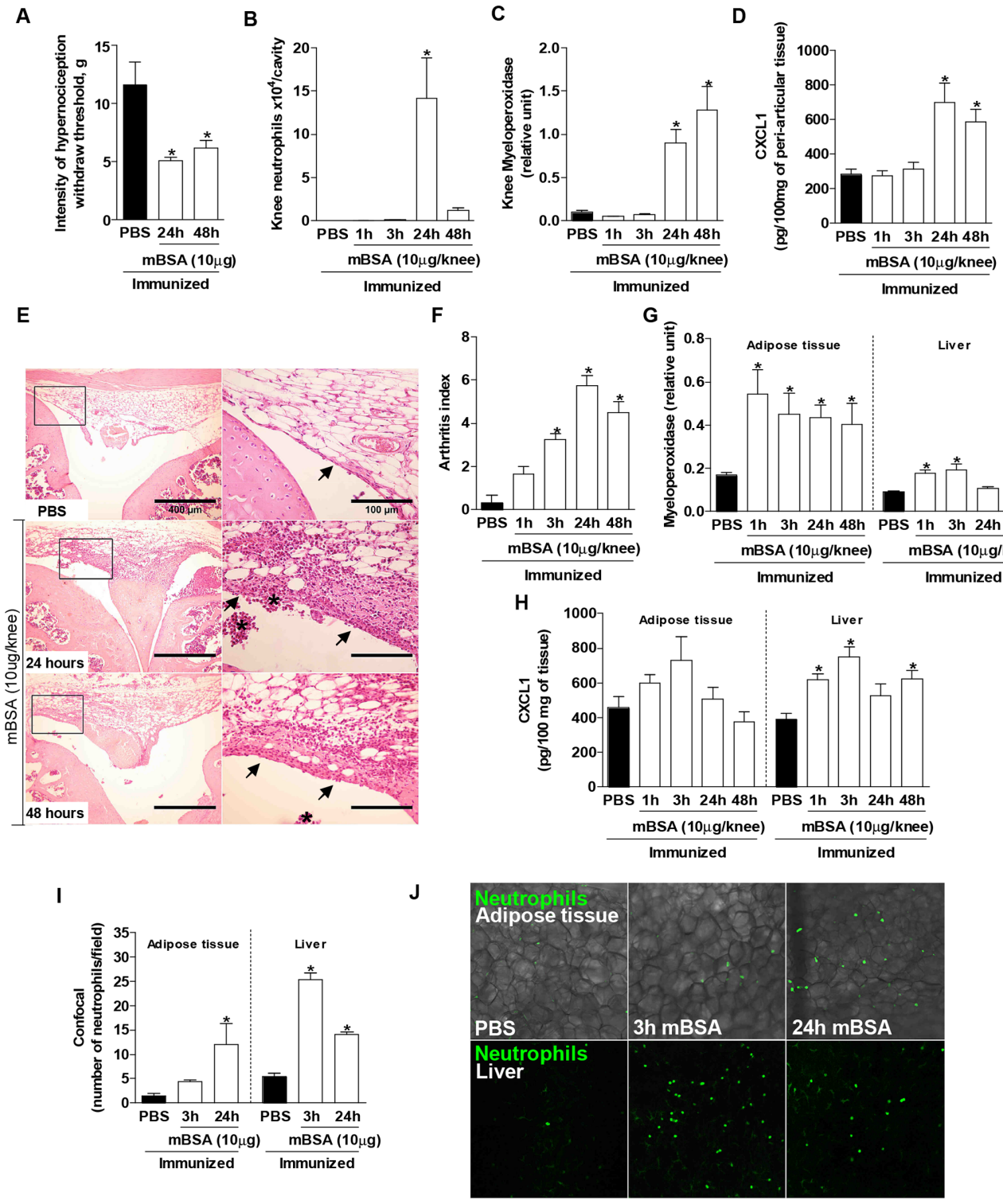

Fig 1. Antigen-induced arthritis in mice is mainly associated with the presence of neutrophils. (A) Intensity of nociception. (B) Number of neutrophils, (C) myeloperoxidase activity, and (D) CXCL1 chemokine levels in the peri-articular tissue. (E) Representative photos of the morphologic alterations in the knee (x100 and x400) and (F) arthritis index. Arrows indicate the synovial and asterisks represent the inflammatory infiltrate. Adipose tissue and liver (G) myeloperoxidase activity, and (H) CXCL1 levels at 1, 3, 24 and 48 hours after the challenge with the antigen-induced arthritis (AIA). Bars represent the mean values±SEM (n = 6–8). Neutrophils number in (I) epididymal adipose tissue and liver accumulated in Lysm-eGFP mice and (J) representative confocal microscopy image, 3 and 24 hours after the challenge with the AIA (x100). Bars represent the mean values±SEM (n = 3–5), *P<0.05 vs. PBS.

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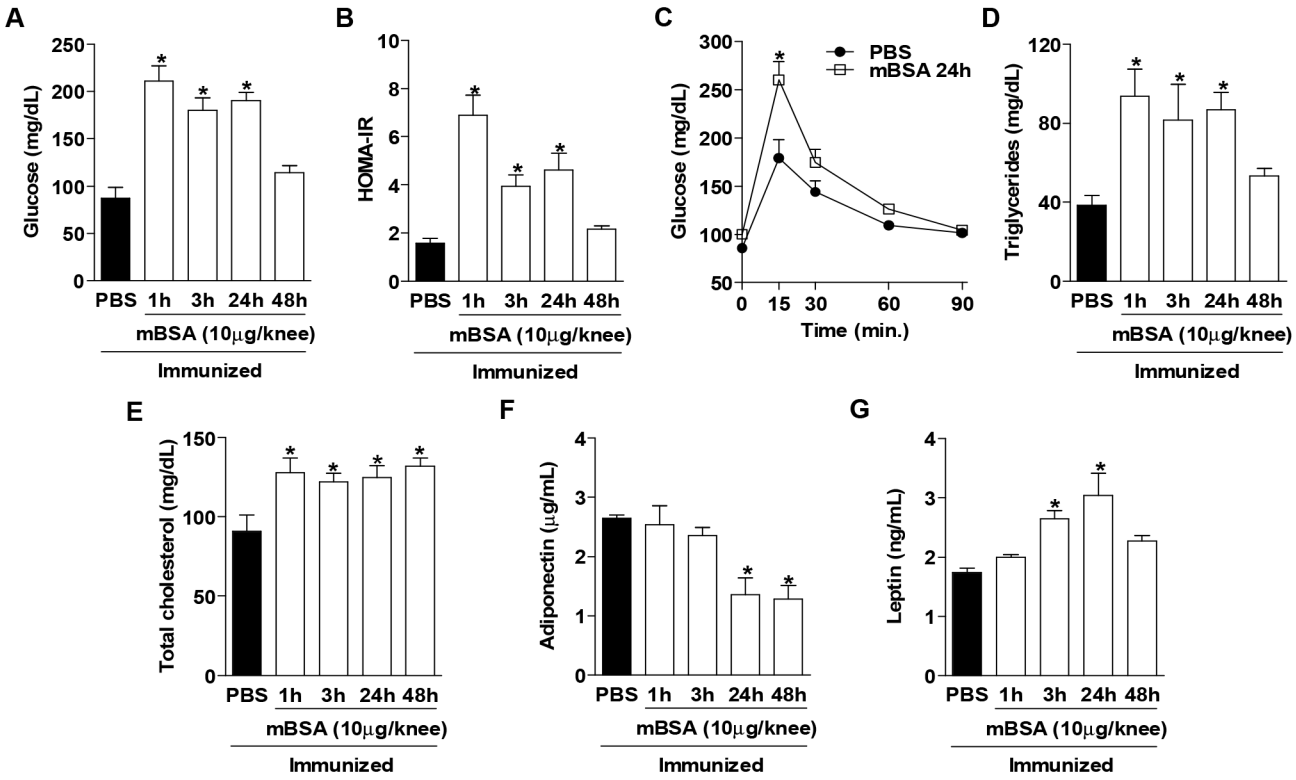

Fig 2. Systemic metabolic alterations in antigen-induced arthritis mice at different time points. The metabolism of glucose showed by (A) glucose levels, (B) HOMA-IR index and (C) oral glucose tolerance test. The lipid metabolism represented by (D) triglyceride and (E) total cholesterol levels. Serum levels of the adipocytokines, (F) adiponectin and (G) leptin at 1, 3, 24 and 48 hours after the challenge with the antigen-induced arthritis (AIA). The bars represent the mean values ± SEM (n = 6–8), **P*<0.05 vs. PBS.

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produced during inflammatory responses [18]. Interestingly, Etanercept also prevented the increase in PTX3 levels observed after induction of AIA (Fig 3K). Therefore, TNF appears to represent an important component for the altered systemic metabolic parameters in this model of AIA.

Although neutrophils are infiltrated into the adipose tissue and liver, they do not appear to alter metabolic parameters induced by AIA

The inflammatory response developed in this model of arthritis is characterized by marked neutrophil influx, neutrophil-dependent joint damage and dysfunction [9]. Next, we investigated whether neutrophils, which were reduced by Etanercept, could contribute to the metabolic changes observed in AIA mice. Mice pre-treated with DF2156A, a CXCR1/2 receptor antagonist, showed reduced intensity of nociception, neutrophil accumulation into the knee joint and in the peri-articular tissue following AIA challenge (Fig 4A–4C), although no alterations in the CXCL1 levels were found (Fig 4D). Joint damage, as assessed histologically was also improved in DF2156A-treated mice (Fig 4E). However, the treatment with DF2156A did not alter changes in glucose levels and HOMA-IR induced by AIA (Fig 4F and 4G), and actually, the glucose intolerance worsened (Fig 4H). Similarly, treatment with DF2156A tended to enhance further the levels of triglycerides and total cholesterol (24 hours) (Fig 4I). Levels of adiponectin were further enhanced at 24 hours by the treatment with DF2156A whereas levels of leptin were similarly increased in DF2156A and vehicle-treated animals (Fig 4J). It was

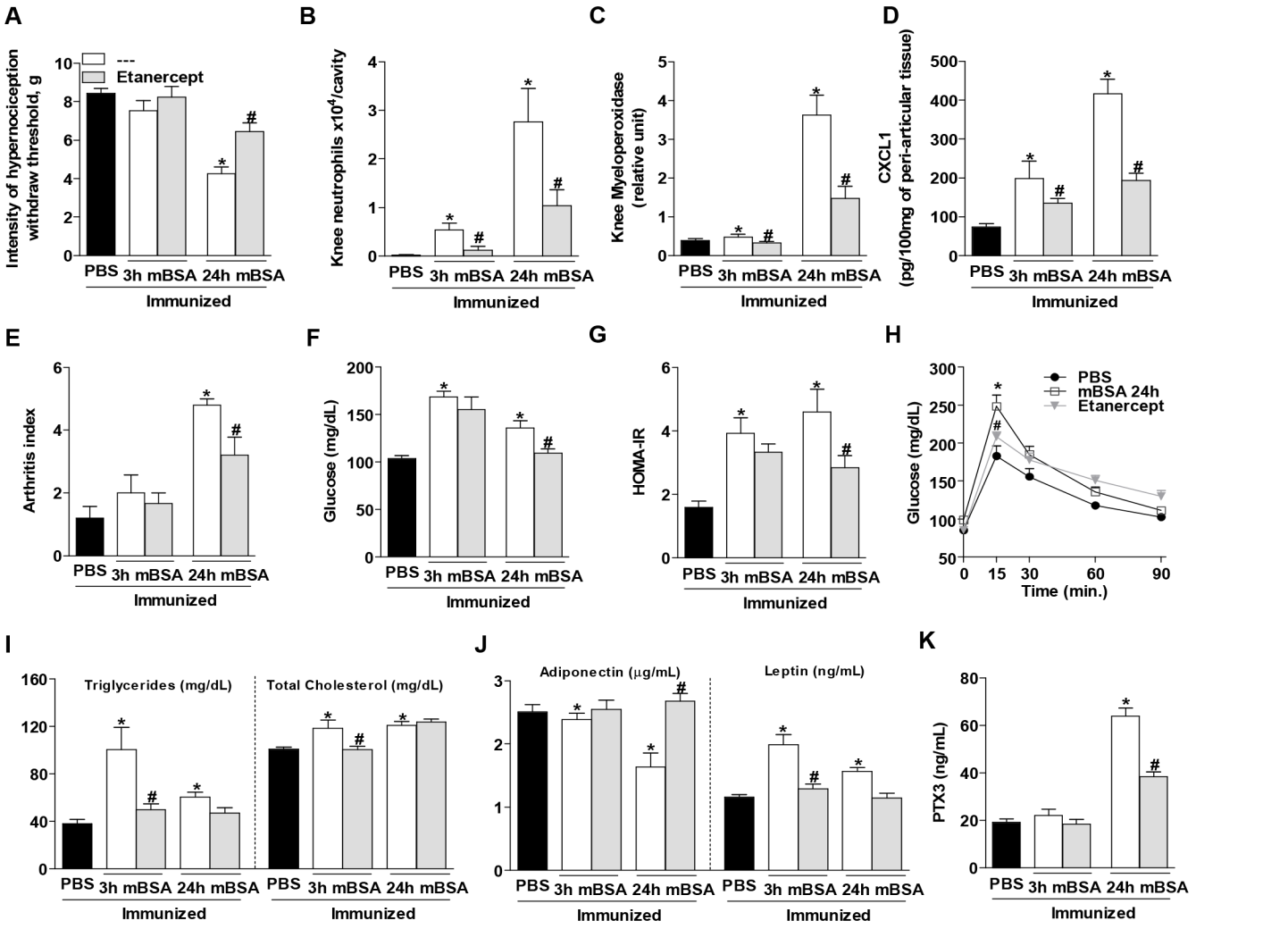

Fig 3. Pre-treatment with Etanercept improves the altered metabolic parameters of antigen-induced arthritis mice. (A) Intensity of nociception. (B) Neutrophils, (C) myeloperoxidase activity, (D) CXCL1 chemokine in the peri-articular tissue and (E) arthritis index. Systemic glucose metabolism showed by (F) glucose levels, (G) HOMA-IR index and (H) oral glucose tolerance test. The lipids, (I) triglyceride and total cholesterol levels. The adipocytokines, (J) adiponectin and leptin levels. (K) PTX3 levels of mice that received a knee intra-articular injection of PBS, mBSA or mBSA treated prior with Etanercept. Bars represent the mean values ±SEM (n = 6–8), **P*<0.05 vs. PBS; #*P*<0.05 vs. AIA of the respective time.

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observed increased PTX3 levels in AIA mice, especially 24 hours after challenge. The treatment with DF2156A induced a further increase in PTX3 levels at both time points evaluated (Fig 4K).

In other to better investigate the role of neutrophils in the systemic metabolic alterations observed in AIA mice, we used the monoclonal antibody RB6-8C5 (anti-GR-1) to deplete neutrophils. We confirmed the depletion of neutrophils in these mice by flow cytometry and differential leukocyte count in the blood (S1 Fig), with no alteration on the absolute amount of lymphocytes or monocytes (data not shown). The depletion was also This treatment was effective in reducing hypernociception (Fig 5A). Corroborating with the efficacy of neutrophils depletion, we did not observe increased neutrophil accumulation into the knee joint or in the peri-articular tissue in RB6-8C5-treated mice after AIA induction (Fig 5B and 5C). Analyses of systemic metabolism showed a similar profile to that seen in DF2156A-treated mice. Again,

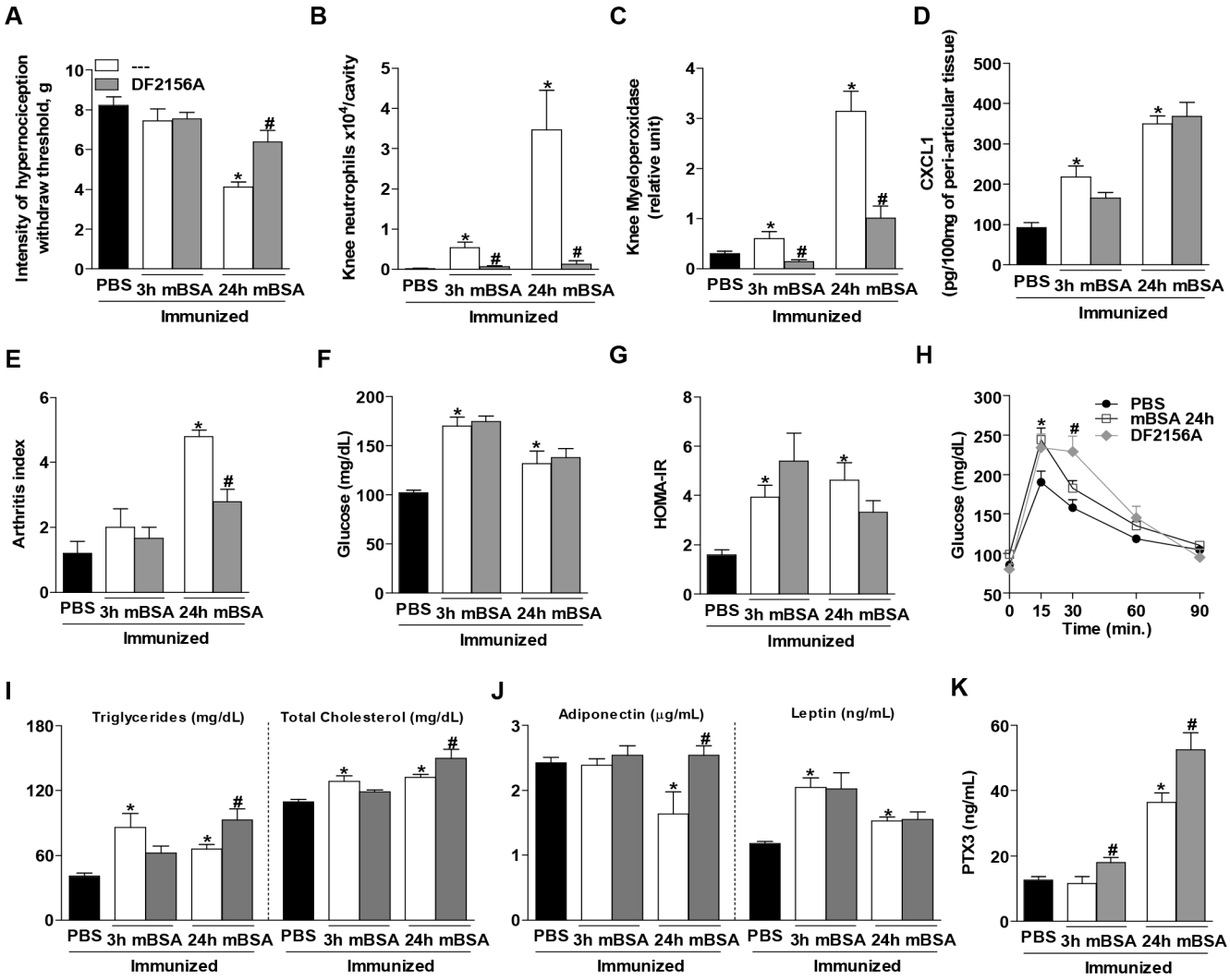

Fig 4. Pre-treatment with DF2156A contributes to metabolic alterations in antigen-induced arthritis mice. (A) Intensity of nociception. (B) Number of neutrophils, (C) myeloperoxidase activity, (D) CXCL1 chemokine in the peri-articular tissue and (E) arthritis index. Systemic glucose metabolism showed by (F) glucose levels, (G) HOMA-IR index and (H) oral glucose tolerance test. The lipids, (I) triglyceride and total cholesterol levels. The adipocytokines, (J) adiponectin and leptin levels. (K) PTX3 levels of mice that received a knee intra-articular injection of PBS, mBSA or mBSA treated prior with DF2156A. Bars represent the mean values±SEM (n = 6–8), *P<0.05 vs. PBS; #P<0.05 vs. AIA of the respective time.

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the neutrophil depletion did not prevent the metabolic dysfunction induced by arthritis. There was no alteration on glucose levels in neutrophil-depleted mice compared with AIA groups (Fig 5D). Moreover, triglyceride and cholesterol levels increased further in neutrophil-depleted mice 3 hours after challenge (Fig 5E). Levels of adiponectin reduced at 24 hours and leptin increased further (Fig 5F) in neutrophil-depleted mice compared with AIA control mice. As observed in DF2156A-treated mice, levels of PTX3 tended to be higher after neutrophil depletion than in control AIA mice (Fig 5G). These observations suggest that the metabolic dysfunction in this model of AIA is not associated with the influx of neutrophils. On the contrary, blockade of neutrophil influx or depletion of neutrophils was associated with a mild worsening of systemic metabolic parameters.

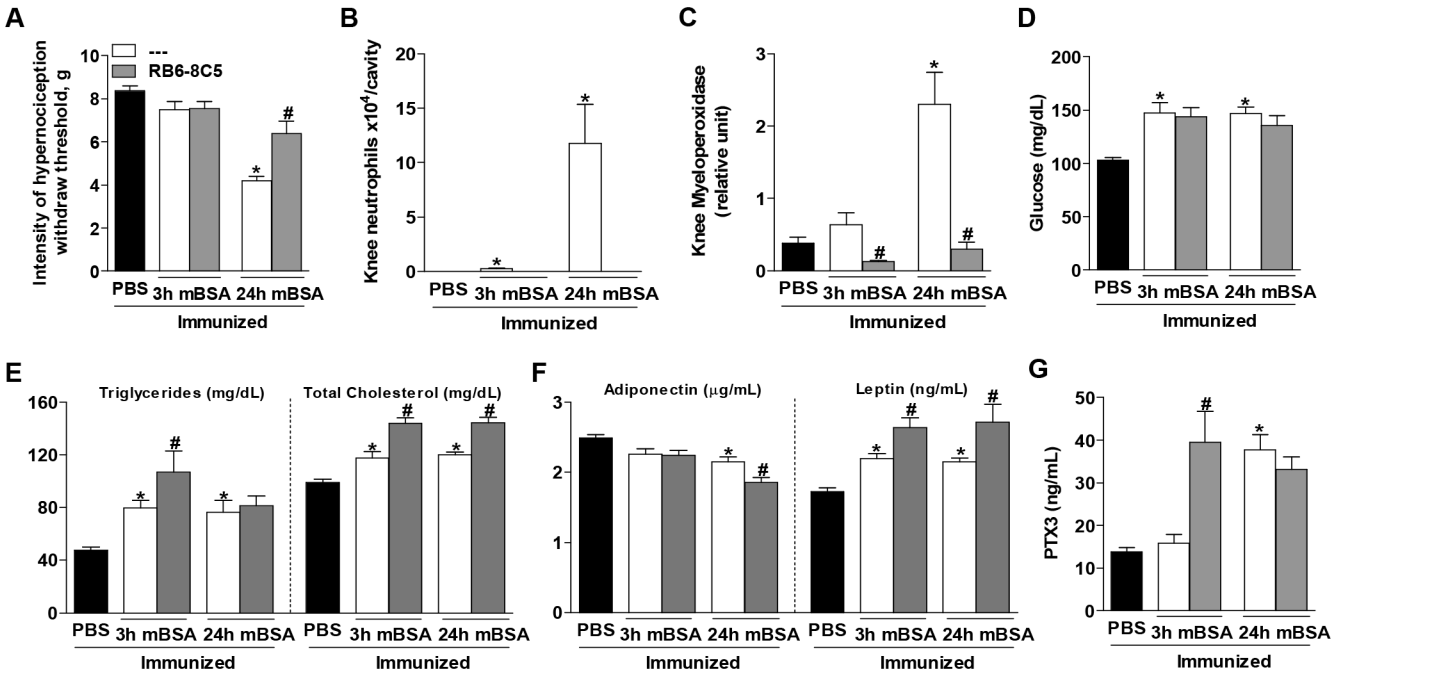

Fig 5. Depletion of neutrophils did not improve the metabolic alterations in antigen-induced arthritis mice. (A) Intensity of nociception. Inflammation in the knee cavity represented by (B) number of neutrophils and (C) myeloperoxidase activity. Metabolic features represented by (D) glucose, (E) triglyceride and total cholesterol levels, accompanied by the adipocytokines (F) adiponectin and leptin levels in the serum. (G) The PTX3 of mice that received an intra-articular injection in the knee cavity of PBS, mBSA or mBSA treated prior with RB6-8C5. The bars represent the mean values±SEM (n = 4–6), *P<0.05 vs. PBS; #P<0.05 vs. AIA of the respective time.

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Discussion

Arthritis is frequently associated with metabolic dysfunction, which may be a consequence of the systemic inflammatory response of this disease [5, 19]. Herein, we have shown that the acute model of AIA transiently alters the metabolism of mice, as observed by higher glucose and lipid levels, altered adipocytokine production and by the increase in neutrophil infiltration into the adipose tissue and liver. TNF appears to be an important mediator of the metabolic changes in AIA mice. The investigation of the mechanisms that lead to metabolic alterations ruled out an important participation of the neutrophils, the main cell type involved in the inflammatory response in this model.

The metabolic syndrome is a risk factor for patients with rheumatoid arthritis (RA) [1] and the cause of this dysfunction is still unclear. Some studies have demonstrated that RA patients present alterations in the glucose and lipid metabolism, including insulin resistance, high levels of cholesterol and hypertriglyceridemia in the absence of obesity [20, 21]. Indeed, we have shown that transient metabolic changes may occur even in lean mice during acute arthritis induction. The inflammation is known to induce an increase in the metabolic demand [22]. This increase appears to be transient, but necessary to provide the appropriate fuel for an acute inflammatory response and subsequent recover to homeostasis. The inflammation observed in the adipose tissue and liver may also trigger the mobilization and systemic release of nutrients, as demonstrated in other acute inflammatory processes [23, 24]. It is also described that the intermediated metabolism (gluconeogenesis and lipolysis) is activated during the process of stress [25], leading to increase in lipids and glucose in the circulation [26, 27]. Moreover, the alteration of these parameters may be a consequence of systemic inflammation [28] and be involved with pain, which is often observed in patients with arthritis [29]. Acute pain by itself

was associated to insulin resistance, increasing serum levels of glucose and free fatty acids in humans [30]. However, there is a lack of studies showing the association between pain and metabolic alterations apart of the context of inflammation.

TNF is a major cytokine in the context of experimental and human arthritis. We showed that anti-TNF therapy (by using Etanercept) successfully reduced the acute metabolic changes after AIA in mice. This is consistent with studies showing that anti-TNF therapy improves the disease and ameliorates the insulin sensibility and lipid levels in RA patients [7, 31]. In rabbits, activated fibroblast-like synoviocytes are important sources of TNF in antigen-induced arthritis model [32], which could also be the main source of TNF in our model. TNF is a cytokine involved in the expression of many other cytokines that act in the host defense, and contributes to the production of acute phase proteins [33, 34]. The PTX3, an acute phase protein, is produced by leukocytes [18] and its synthesis is induced by cytokines such as TNF and IL-1β [35, 36]. This axis appears to be important to trigger the metabolic changes observed in our study. In fact, mice treated with anti-TNF showed low levels of PTX3 along with some improvement on metabolic changes. In addition to contribute to acute inflammation, there are much data demonstrating that TNF also participates in the resolution of inflammation and tissue remodeling [37]. These latter effects of TNF may be relevant in chronic RA patients as tissue remodeling may help adapting to the chronic inflammatory stimulation and chronic metabolic stress.

TNF is also synthesized in the adipose tissue and may act on glucose metabolism through the increase in lipolysis [38], and consequently contribute to insulin resistance [39]. This cytokine may also alter the production of adipocytokines, which are involved in the inflammatory process as well as metabolism [40]. Adiponectin is considered an anti-inflammatory cytokine and is associated to the insulin sensitivity [41]. Controversially, patients with RA showed an increase in adiponectin levels that are associated to the disease progression [42]. The decrease in this cytokine observed in our model may be due to the acute inflammation induced by the arthritis, yielding the insulin insensitivity. Leptin, which is a pro-inflammatory adipocytokine, is also increased in RA patients as a predictor of disease duration and development [43]. Although leptin may contribute to the systemic inflammation and consequently metabolic alterations, the higher levels of this cytokine following AIA may be also a consequence of the acute inflammatory response. Indeed, recent findings showed that leptin secretion from the adipose tissue is induced few hours after LPS i.p. injection [37]. Combined, these data suggest that metabolic changes appear to be linked to the acute increase of pro-inflammatory mediators rather than neutrophils. Moreover, our data suggest that TNF is a major driver of adipocytokines in the context of acute inflammation. Therefore, it appears that inflammatory mediators are the primary factor driving systemic metabolic changes in arthritis.

Neutrophils and cytokines have a critical role in the inflammatory response observed in RA and in different models of arthritis [44, 45]. A possible explanation for the accumulation of neutrophils in adipose tissue and liver following the challenge into the knee could be related to soluble mediators released by joint resident cells, such as TNF, although we could not detected it in serum. Moreover, the previously migrated neutrophils into the joint could reach the circulation again [46], a phenomenon called reverse transmigration, which, in turn, could accumulate in other organs, as observed in adipose tissue and liver. In the context of RA, the role of neutrophils is more controversial since these cells are not majority in patients with severe disease. However, there is evidence that neutrophil influx does occur during recurrence of disease and may contribute to joint damage [4]. It is known that neutrophils contribute to the production of cytokines [47], reactive oxygen species [48] in an acute inflammatory response. Neutrophil may also contribute to metabolic changes including insulin resistance and liver steatosis, induced by obesity [49, 50]. In our experiments, the use of a CXCR1/2 antagonist did not improve the systemic metabolic alterations observed in AIA mice. However, we did have

concerns whether these results were due to the presence of some neutrophils still activated in the circulation. Thus, we depleted neutrophils. Again, the acute systemic metabolic alterations of AIA mice were still present, even in the absence of neutrophils. Moreover, the levels of PTX3 were even increased after the inhibition of neutrophil recruitment or their depletion. Therefore, the worsening of some metabolic parameters in AIA mice may be associated to the increase in systemic inflammatory response that appears to be involved in the production of cytokines.

In summary, our data showed that the induction of local joint inflammation in mice provoked acute alterations in systemic metabolic parameters that were accompanied by an influx of neutrophils into the adipose tissue and liver. The cytokine TNF appears to significantly contribute to the acute systemic metabolic changes. However, the influx of neutrophils does not account for the metabolic changes observed after AIA. We suggest that the inflammatory cytokines may be the main factors involved in the improvement of the altered systemic metabolic parameters induced by anti-TNF therapy.

Supporting Information

S1 Fig. The depletion of neutrophils in the blood of mice treated with RB6-8C5 MAb clone. Analysis of blood cells in the mice that received an intra-articular injection in the knee cavity of PBS, mBSA or mBSA treated prior with RB6-8C5. It is represented the absolute number of (A) total leukocytes and (B) neutrophils. (C) Frequency of total neutrophils ($GR1^{+high}$ $CD11b^{+high}$) by flow cytometry. The bars represent the mean values±SEM (n = 4–6). (D) Representative dot plots of neutrophils as frequency of parent ($GR1^{+high}$ $CD11b^{+high}$). $^{*}P<0.05$ vs. PBS; $\#P<0.05$ vs. AIA of the respective time.
(TIF)

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Author Contributions

Conceived and designed the experiments: MCO ATV LPS MMT FAA AVMF. Performed the experiments: MCO LPT JPV NVB CMQ ATV GBM FAA. Analyzed the data: MCO LPT JPV NVB CMQ ATV GBM FAA. Contributed reagents/materials/analysis tools: FAJL LPS MMT FAA AVMF. Wrote the paper: MCO FAJL LPS MMT FAA AVMF.

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Chapter 3

Two opposite extreme of adiposity similarly reduce inflammatory response of antigen-induced acute joint inflammation

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Two opposite extremes of adiposity similarly reduce inflammatory response of antigen-induced acute joint inflammation

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ABSTRACT

Objective: Acute inflammation is a normal response of tissue to an injury. During this process, inflammatory mediators are produced and metabolic alterations occur. Adipose tissue is metabolically activated, and upon food consumption, it disrupts the inflammatory response. However, little is known about the acute inflammatory response in joints that results from diet-induced adipose tissue remodeling. The objective of this study was to determine whether alterations in adipose tissue mass arising from food consumption modify the inflammatory response of antigen-induced joint inflammation in mice.
Methods: Male BALB/c mice were fed a chow diet, a highly refined carbohydrate-containing (HC) diet for 8 wk. They were then immunized and, after 2 wk, received a knee injection of methylated bovine serum albumin (mBSA). They were sacrificed at 6, 24, and 48 h after injection. The effect of the cafeteria diet for 8 wk, which also increases adipose tissue, or conjugated linoleic acid (CLA) supplementation for 4 wk, a model of lipodystrophy, was evaluated 24 h after knee challenge with mBSA.
Results: Cellular influx, predominantly neutrophils, in synovial fluid was attenuated in the HC diet group, as were levels of myeloperoxidase and IL-1β in periarticular tissue and histopathological analysis. These responses were associated with reduced adiponectin and increased leptin in serum, which was pronounced in mice fed the HC diet. Cafeteria diet and CLA supplementation induced a profile similar to that seen with the HC diet in terms of inflammation, disease response, and metabolic alteration. Interestingly, after the injection of mBSA, the area of adipocytes in the infrapatellar fat pad increased in mice fed with chow diet similar to those fed the HC and cafeteria diet.
Conclusions: We demonstrated that attenuation of joint response induced by diet was independent of adipose tissue remodeling but could be associated with metabolic alterations.

Introduction

Inflammation is a biological reaction of the tissue to harmful stimuli characterized by the activation of immune system components [1]; when inflammation is persistent, it can lead to

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tissue damage [2]. Importantly, metabolic support and the redistribution of energy are required during an inflammatory response [3]. In particular, there is a rapid mobilization of lipid stocks from specific metabolic organs; this is essential to cellular activation and tissue response [4]. Indeed, nutrients play an important role in supporting the inflammatory process; also, nutrients themselves can trigger inflammation [5,6]. Therefore, changes in the composition of diet can influence the quality of the inflammatory response.

Excessive dietary intake of lipids [5] and carbohydrates [6] can lead to the accumulation of body fat. The main tissue capable of dealing with the resulting excess of energy is adipose tissue, which is metabolically active and capable of remodeling (increasing or decreasing in size) [7,8]. Its expansion, as observed in obesity, results in the recruitment of immune cells to this tissue [9] and contributes to rising levels of serum markers of inflammation [10]. On the other hand, systemic inflammation can also alter the optimal functioning of adipose tissue by increasing lipolysis and leading to insulin resistance [11]. Interestingly, we have shown that the induction of local acute joint inflammation in mice leads to acute alterations in systemic metabolic parameters associated with higher neutrophil influx in the adipose tissue [12]. Further, we hypothesize that any alteration in adipose mass or function could modulate the acute joint inflammatory response in mice.

In this study, we evaluated the effect of diet-induced adipose tissue remodeling on acute arthritic response by using different diet compositions in a model of acute antigen-induced arthritis (AIA) in mice. We demonstrated that the knee inflammatory response is attenuated in distinct models of adipose remodeling (i.e., obesity induced by diet or lipodystrophy).

Materials and methods

Animals and diets

Male BALB/c mice (6 to 7 wk old) were obtained from the animal care center at Universidade Federal de Minas Gerais. They were maintained in an environmentally controlled room under a 12/12-hour light-dark cycle. Animals had free access to tap water and food. The experimental protocol was approved by the Ethics Committee in Animal Experimentation at Universidade Federal de Minas Gerais (protocol: 148/2012).

Mice were fed a standard chow (LABINA) diet, a highly refined carbohydrate-containing (HC) diet, or a cafeteria (CAF) diet for 8 wk. They were immunized with antigen at week 6 and given an injection of antigen at the knee at week 8. The composition of chow diet was 4.0 kcal/g, 65.8% carbohydrate, 3.1% fat, and 31.1% protein. The composition of the HC diet was 4.4 kcal/g, 74.2% carbohydrate (at least 30% refined sugars), 5.8% fat, and 20% protein [5]. The composition of CAF diet was potato chips, chocolate, cookies, peanuts, condensed milk, butter, and soft drinks in addition to standard chow diet. Its nutritional composition was calculated according to the product manufacturers ($\cong$ 5.8 kcal/g, 31% carbohydrate, 58% fat, and 11% protein). In addition, a diet supplemented with conjugated linoleic acid (CLA; 1% w/w of the diet) was used (General Nutrition, Pittsburgh, PA) to induce a phenotype similar to that seen in lipodystrophy [13]. The composition of the CLA-supplemented diet was 4.2 kcal/g, 71.6% carbohydrate, 3.5% fat, and 24.9% protein. The majority of its composition was a 50:50 mixture of 2 isomers, cis-9, trans-11 and trans-10, cis-12. Mice were fed the chow diet for 4 wk and then a CLA-supplemented diet until week 8. They were immunized at week 6 and received the knee challenge at week 8.

Mice were weighed once a week, and the food intake was measured every 2 to 3 d. After each experimental procedure, the animals were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) and sacrificed. Samples of blood were collected, as were samples of tissue from the knee. Epididymal, retroperitoneal, and mesenteric white adipose tissues were collected, weighed, and used to determine the adiposity index (i.e., the sum of epididymal, retroperitoneal, and mesenteric tissue in terms of weight in grams: [g] $\div$ body weight $\times$ 100).

Arthritis induction and determination of articular inflammation

Mice were immunized intradermally with 500 μg of methylated bovine serum albumin (mBSA) in 100 μL of a saline emulsion and an equal volume of complete Freund's adjuvant (CFA). Fourteen days after immunization, they were challenged with an intra-articular injection in the knee joint (10 μg mBSA in 10 μL phosphate-buffered saline [PBS]) [14].

To determine the presence of articular inflammation, the knee cavity was washed with PBS (2 $\times$ 5 μL) for cell recovery. The total number of leukocytes was determined by staining them with Turk's solution and then counting them. Differential counts were obtained from cytospin preparations stained with May-Grünwald-Giemsa stain.

Hypernociceptive assessment

Hypernociception was assessed as previously described [15]. Briefly, mice were placed in acrylic cages with a wire grid floor. A series of stimuli were administered with an electronic pressure meter (INSIGHT Instruments, Ribeirão Preto, São Paulo, Brazil) in the hind paw to induce flexion of the knee joint, followed by withdrawal of the paw. The intensity of the pressure was recorded. The response value was obtained by averaging of 2 measurements for each animal. The intensity of hypernociception is reported as paw withdrawal threshold (in grams); that is, the faster withdrawal represents less force tolerated, indicating higher hypernociception.

Histopathological analysis

Knee joints were fixed in formalin, decalcified (EDTA 14%), and embedded in paraffin. Sections (measuring 7 mm) were stained with hematoxylin and eosin. Two sections/knee joints of each animal were examined and scored in a blind manner as follows: severity of synovial hyperplasia (0–3), intensity and extension of inflammatory infiltrate (0–4), and bone erosion (0–2). The grades were summed to obtain the arthritis index (0–9).

For the analysis of infrapatellar adipocytes, images of 6 fields from each animal were captured using a microscope ($\times$100) coupled to a camera. The cell area was measured in each animal by using ImageJ software (National Institutes of Health, Bethesda, MD). The number of infrapatellar adipocytes was counted in an area of 200 μm^2.

Myeloperoxidase activity

Indirect neutrophil presence in the knee was measured by assaying myeloperoxidase activity, as described previously [16]. Briefly, the periarticular tissue (knee joint) was homogenized and assayed for myeloperoxidase activity by measuring the change in OD (450 nm) using tetramethylbenzidine as substrate.

ELISA assay

IL-1β was determined in the periarticular tissue; adipocytokines, adiponectin, resistin, and leptin were analyzed in the periarticular tissue and serum using DuoSet ELISA development kits (R&D System, Inc., Minneapolis, MN) according to the manufacturer's instructions.

Lysate preparation and Western blot analysis

Inflammatory cells harvested from the knee cavity were washed with PBS and whole cell extracts were obtained by using a lysis buffer (1% Triton X-100, 100 mM Tris/HCl, pH 8.0, 10% glycerol, 5 mM EDTA, 200 mM NaCl, 1 mM DTT, 1 mM PMSF, 25 mM NaF, 2.5 μg/mL leupeptin, 5 μg/mL aprotinin, and 1 mM sodium orthovanadate). Lysates were centrifuged and quantified using the Bradford assay reagent from Bio-Rad (Hercules, CA). Extracts (20 μg) were separated by electrophoresis on a denaturing 10% SDS-PAGE and transferred onto nitrocellulose membranes as described [17]. Membranes were blocked overnight with 5% (w/v) nonfat dry milk and then incubated with primary antibodies from Cell Signaling Technology (Beverly, MA) using a dilution of 1:1000. After washing, membranes were incubated with horseradish peroxidase–conjugated secondary antibody (1:3000). Immunoreactive bands were visualized by using the ECL detection system (GE Healthcare, Piscataway, NJ).

Statistical analysis

Results were expressed as mean $\pm$ SEM. Multiple comparisons were performed using one-way analysis of variance. However, the post hoc tests differed according to the experimental protocol. In the kinetic experiment, comparisons of the same diet given at different times were used with Dunnett's post hoc test; testing was also done at the same time point to compare different diets (the chow and HC diets) using Student's t test. For experiments with 4 different diet compositions, the Newman-Keuls post hoc test was used. Two-way analysis of variance was performed to arrive at body weight gain data, followed by the Bonferroni post hoc test. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, CA). Statistical significance was set at $P < 0.05$.

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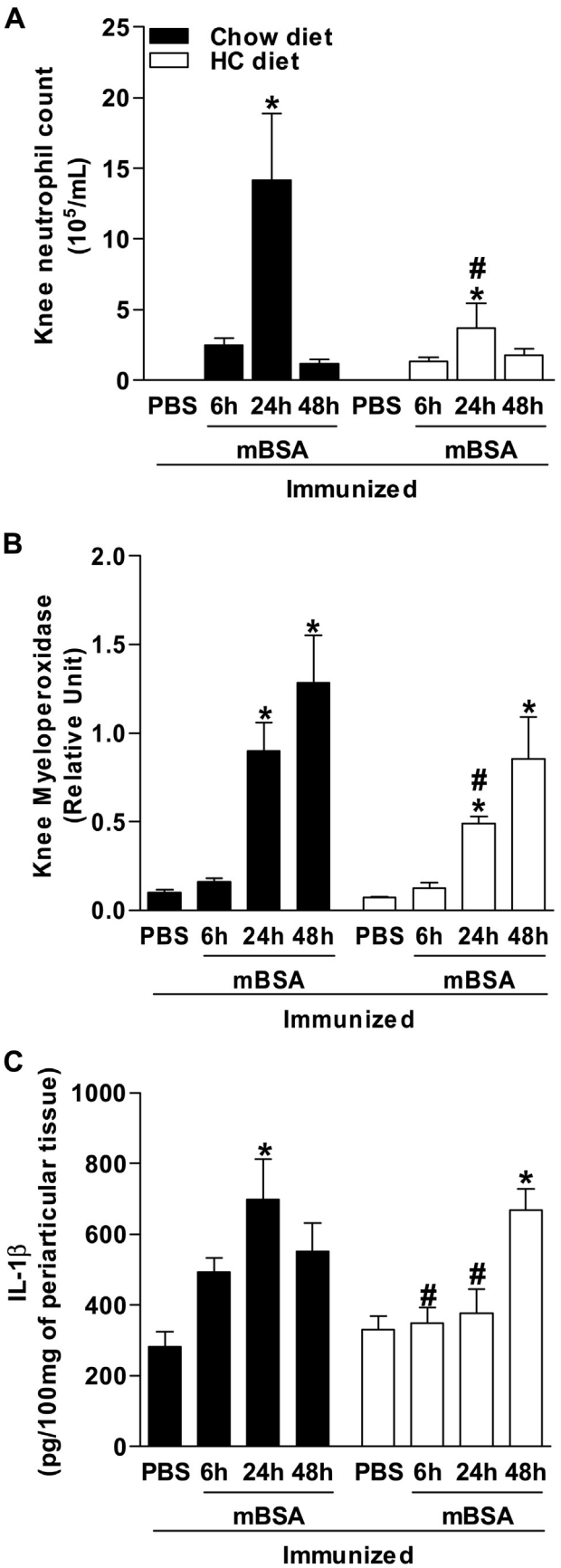

Results

HC diet attenuates joint inflammation and alters adipocytokines in serum upon antigen challenge

The recruitment of neutrophils into the synovial cavity (Fig. 1A) and periarticular tissue (Fig. 1B) peaked at 24 h after challenge. However, compared with the chow diet, the HC diet reduced the accumulation of neutrophils in both compartments (Fig. 1A, B), in association with decreasing IL-1β levels in the periarticular tissue (Fig. 1C).

At 6, 24, and 48 h after mBSA challenge, the chow diet group showed a significant change in hypernociception (decreased paw withdrawal threshold, an index of pain) compared with the control PBS group, which had a response similar to that in the HC diet groups (Fig. 2A). Histopathological analysis showed an intense infiltration of neutrophils and increased synovial hyperplasia, albeit without bone erosion in mice fed the chow diet. This response was attenuated at 6 and 24 h in the mice fed the HC diet (Fig. 2B, C).

We recovered whole cells from the synovial fluid of mice and processed them for the evaluation of intracellular signaling molecules. According to the reduced inflammatory response, at 24 h after mBSA challenge, p-ERK1/2 and p-IκB-α levels were lower in the group receiving the HC diet compared with control arthritic mice (Fig. 3A, B). Interestingly, the challenge with mBSA increased both intact and cleaved forms of Annexin-A1 (AnxA1) compared with PBS-challenged mice. Mice on the HC diet had a higher intact form of AnxA1 in the synovial cells at the peak of inflammation (24 h) compared with mice fed the chow diet (Fig. 3A, B).

Adiponectin, leptin, and resistin are adipocytokines mainly released by adipose tissue and associated with the development of rheumatoid arthritis [18]. In our study, serum adiponectin levels decreased in arthritic mice compared with nonarthritic mice throughout the experimental period independent of diet composition (Fig. 4A). As expected, the HC diet reduced serum adiponectin to basal levels compared with the chow diet. This reduction was maintained after mSBA challenge (Fig. 4A). On the other hand, serum leptin levels increased after mBSA challenge, mainly at earlier time points (Fig. 4B). The HC diet also increased leptin to basal levels and increased further only at 6 h after arthritis induction (Fig. 4B). There were no significant changes in the levels of serum resistin in the chow diet groups; there was a reduction at 24 h after mBSA challenge only in mice fed the HC diet compared with nonarthritic fed the same diet (Fig. 4C).

Distinct diet-induced adipose tissue remodeling also decreases joint inflammation in mice

Because an attenuation of the knee inflammatory response was observed in the HC diet group, we investigated whether other types of diet-induced adipose tissue remodeling could also play a role in arthritic mice. For this, mice were fed the CAF diet or a diet supplemented with CLA. There were no differences in food intake between the diet groups (chow diet: 4.6 ± 0.11 g/d per mouse; HC

Fig. 1. Inflammation is attenuated in mice with antigen-induced joint inflammation after being fed a highly refined carbohydrate-containing (HC) diet. (A) Neutrophils in the synovial cavity and (B) myeloperoxidase activity in periarticular tissue. (C) IL-1 β levels in the periarticular tissue of mice fed chow or an HC diet at 6, 24, and 48 h after antigen challenge in the joint. Bars represent the mean ± SEM (n = 6–8). *$P < 0.05$ versus PBS; #$P < 0.05$ versus chow diet of the same time.

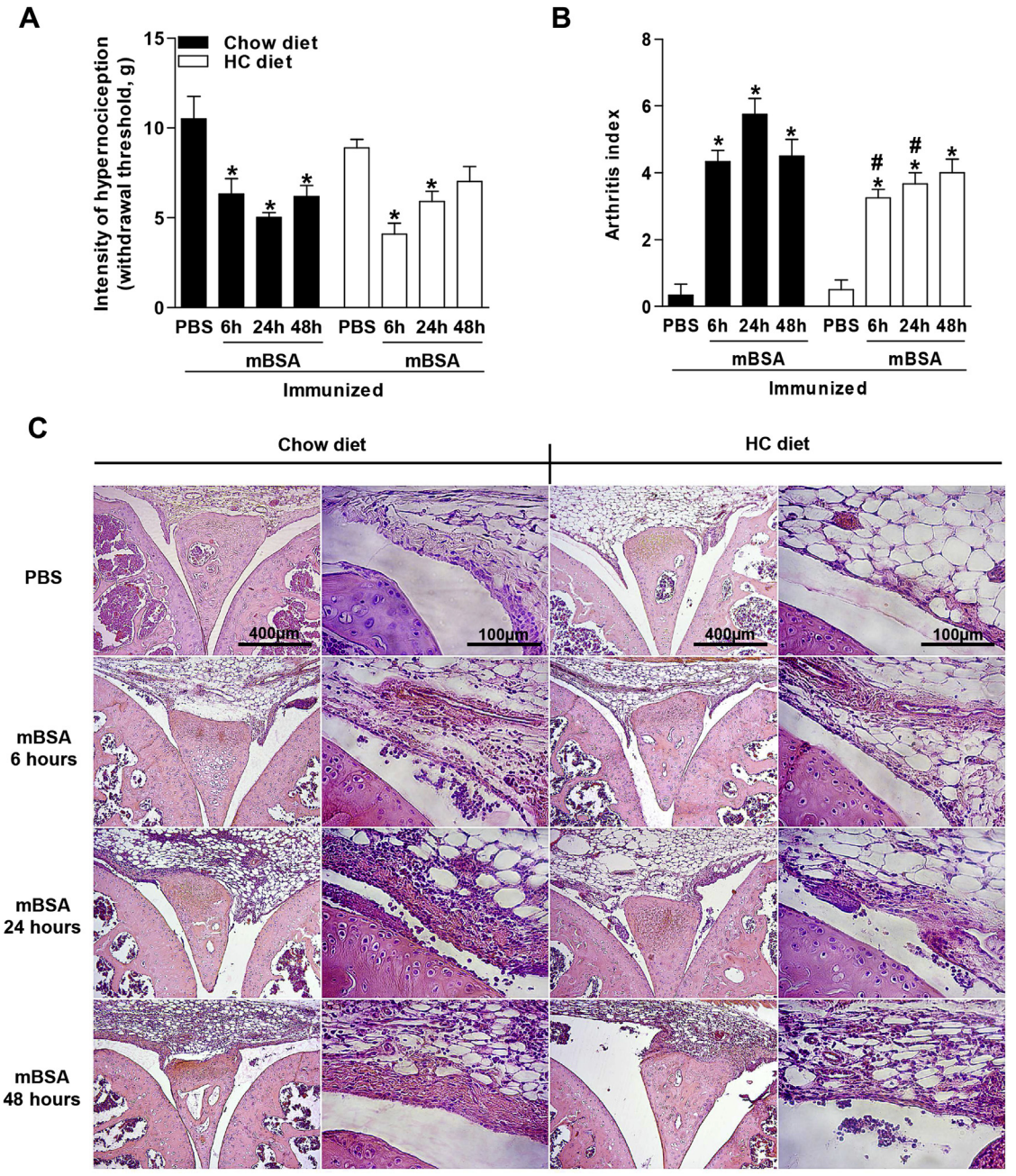

Fig. 2. Nociception assay and morphologic alterations in the knees of mice fed chow or a highly refined carbohydrate-containing (HC) diet. (A) Intensity of nociception by Von Frey filaments. (B) Arthritis index. (C) Representative photos of the morphologic alterations in the knee (×100 and ×400) at 6, 24, and 48 h after antigen challenge in the joint. Bars represent the mean ± SEM ($n = 6–8$). *$P < 0.05$ versus PBS; #$P < 0.05$ versus chow diet of the same time.

diet: 4.9 ± 0.11 g/d per mouse; CAF diet: 5.1 ± 0.14 g/d per mouse; CLA diet: 4.8 ± 0.12 g/d per mouse). However, body weight increased more in mice fed the CAF diet and was lower in mice supplemented with CLA compared with the chow diet (Fig. 5A). Although the HC diet did not affect body weight, it increased the adiposity index even 24 h after antigen challenge (Fig. 5B). Like the HC diet, the CAF diet also increased the adiposity index in arthritic mice. On the other hand, there was a reduction in the adipose mass of the CLA-supplemented group (Fig. 5B). When challenged with mBSA, chow diet mice showed increased glucose levels compared with the PBS group; that is, glucose levels were higher in the HC diet group compared with the AIA control group (Fig. 5C). We also analyzed the number and extent of infrapatellar adipocytes around the knee. As seen in Figure 5D, hypertrophy of infrapatellar adipocytes was noted in AIA mice fed the chow diet compared with nonarthritic mice. This increase was similar in arthritic mice fed the HC or CAF diets (Fig. 5D). However, AIA mice fed the CLA-supplemented diet had a smaller adipocyte area compared with control AIA mice (Fig. 5D). The number of adipocytes in the same area was reduced in the AIA control group compared with nonarthritic mice, as also observed in mice on the HC and CAF diets but not in those on the CLA-supplemented diet (the number of adipocytes in mice on the various diets were as follows: PBS, 67.1 ± 2.3; chow diet, 43.7 ± 2.2; HC diet, 43.4 ± 3.2;

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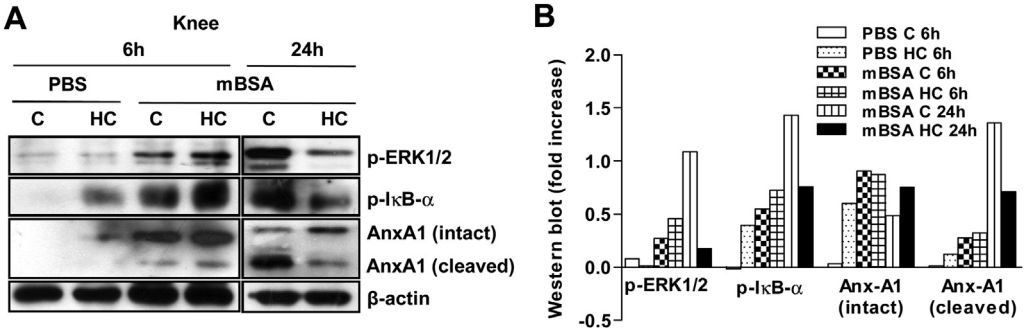

Fig. 3. Western blot analysis showing the effect of a highly refined carbohydrate-containing (HC) diet on the inflammatory response in synovial fluid. (A) Representative blots of synovial fluid cells of mice fed chow or the HC diet at 6 and 24 h after antigen challenge in the joint. Extracts (20 μg) were collected and processed for analysis of p-ERK1/2, p-IκB-α, Annexin A1 (AnxA1), and β-actin levels. (B) Fold increase of p-ERK1/2, p-IκB-α and AnxA1 levels in relation to β-actin.

CAF diet, 38.0 ± 3.9; CLA diet, 10.0 ± 1.7). Interestingly, independent of HC, CAF, or CLA-supplemented diets, there was a reduction in the number of neutrophils recovered from the synovial cavity in all of these groups (Fig. 5E) and tissue lesion (clinical score; Fig. 5F) compared with arthritic mice fed the chow diet. Figure 5G is representative of adipocytes in the knee.

Because adipocyte size was altered upon AIA response, adipocytokines levels in the serum and periarticular tissue were evaluated. Adiponectin and resistin were reduced and leptin was increased in AIA mice fed the chow diet compared with control PBS in the serum (Fig. 6A–C). The HC diet group showed a reduction in adiponectin levels (Fig. 6A) and the CAF diet increased only adiponectin (Fig. 6A) compared with chow diet AIA mice; however, both were without differences in leptin levels in the serum (Fig. 6B). Adiponectin was reduced but leptin and resistin levels were increased in AIA mice fed the chow diet compared with control PBS in periarticular tissue (Fig. 6D–F). Mice fed the HC and CAF diets presented no differences compared with chow diet mice challenged with antigen in the same tissue (Fig. 6D–F). All adipocytokines levels were reduced in CLA-supplemented mice in the serum or periarticular tissue (Fig. 6A–C, E, F), except for adiponectin in the joint, which was increased (Fig. 6D).

Discussion

Main findings

Adipose tissue is a key metabolic organ and variations in its size or composition may lead to metabolic and inflammatory diseases [5,6]. Our aim in the present study was to determine whether the remodeling of adipose tissue mass affects the acute inflammatory response in the joint. We demonstrated that the HC, CAF, and CLA-supplemented diets decreased acute inflammation after antigen challenge by reducing the accumulation of neutrophils in the knee cavity and, thus, tissue damage as well. Moreover, adipocytes present in the infrapatellar knee were hypertrophied after antigen challenge in control mice similarly to those fed HC and CAF diets, which was associated with the altered production of adipocytokines.

Major implications

Metabolic and inflammatory disorders resulting from the accumulation of body fat can be initiated and maintained mainly by the intake of saturated fatty acids [5] and refined carbohydrates [6]. Previous results from our group showed that mice fed the HC diet exhibited increased adiposity but no significant difference in weight gain [6]. However, the CAF diet was shown to increase both [19]. The alteration in the volume of adipose tissue in these mice can modulate the inflammatory response by worsening it, as demonstrated in obese mice [20], or in humans by increasing the risk and severity of acute [21] and chronic [22] inflammatory diseases. In our study, we first expected an increase in severity of joint inflammation in mice fed the HC and CAF diets. However, we observed an opposite effect (i.e., independent of whether the diets increased both adiposity and weight or only adiposity). In accordance with our findings, some studies have shown that obesity also plays a dual role in reducing joint destruction in rheumatoid

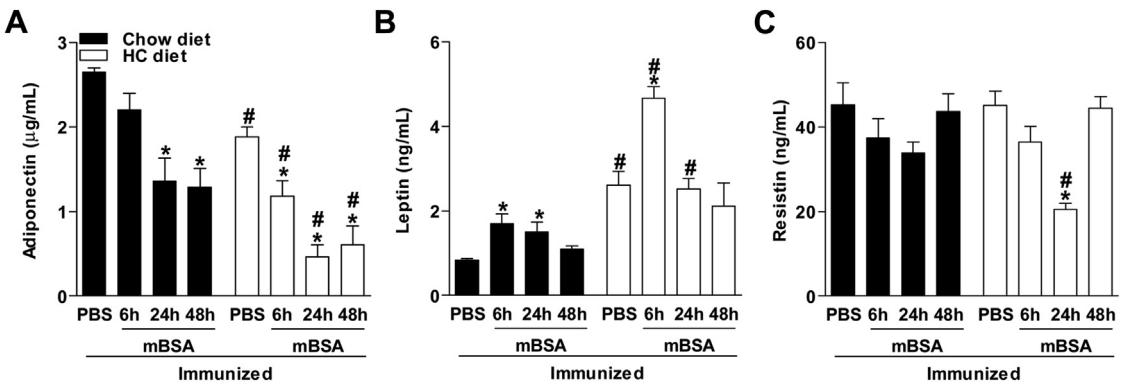

Fig. 4. Systemic alteration of adipocytokines in mice with antigen-induced arthritis at different time points. (A) Adiponectin, (B) resistin, and (C) leptin levels in the sera of mice fed chow or a highly refined carbohydrate-containing (HC) diet at 6, 24, and 48 h after antigen challenge in the joint. Bars represent the mean ± SEM (n = 6–8). *$P < 0.05$ versus PBS; #$P < 0.05$ versus chow diet of the same time.

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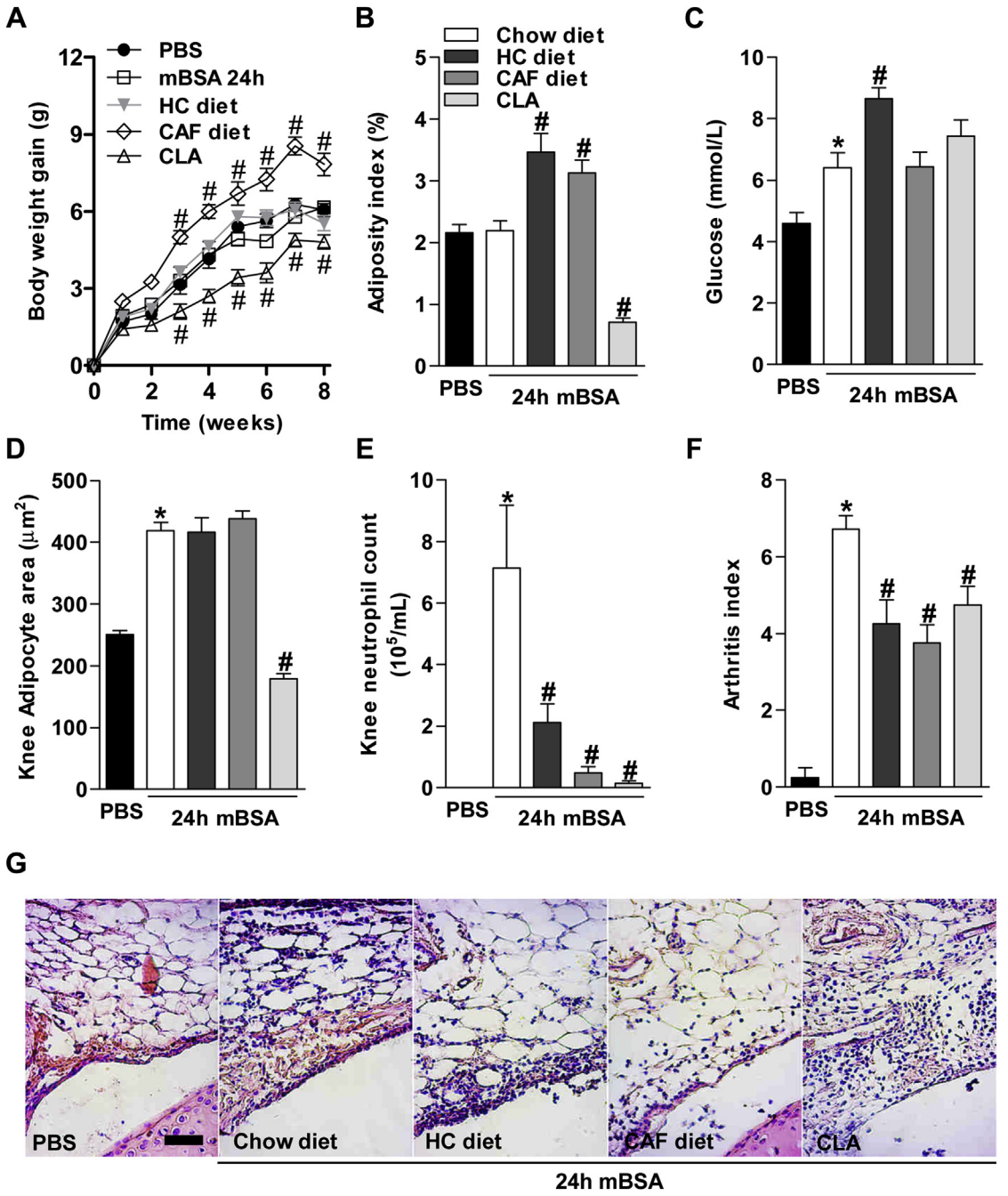

Fig. 5. Inflammatory infiltrate is attenuated in mice with antigen-induced joint inflammation that were fed a highly refined diet supplemented with carbohydrate (HC), cafeteria (CAF) diet, and conjugated linoleic acid (CLA)-supplemented diet. (A) Body weight gain. (B) Adiposity index. (C) Serum glucose levels. (D) Infrapatellar adipocyte area. (E) Neutrophils in the synovial cavity. (F) Arthritis index of mice fed chow, HC, CAF and CLA-supplemented diet at 24 h after antigen challenge in the joint. Bars represent the mean ± SEM (n = 7–8). *$P < 0.05$ versus PBS; #$P < 0.05$ versus chow diet mBSA group. (G) Representative photos of the infrapatellar adipocytes in the knee (×100). Bar indicates 50 μm.

arthritis or protecting against osteoporosis [23,24]. In addition, mice fed a high-fat diet during 12 wk in an acute model of gout did not manifest an exacerbated inflammatory response after intraperitoneal injection of monosodium urate crystals [25]. Similarly, consumption of a high-carbohydrate diet for 8 wk by food-allergic mice did not lead to an exacerbation of the pathologic response [26]. These studies indicate that adipose tissue can also play a protective role, probably driven by hormones, in the inflammatory response.

Because we observed an attenuation of inflammation in the knees of mice fed the HC and CAF diets, which typically expand adipose tissue, we used a dietary component that could reduce it. We expected a worsening of the knee response. Surprisingly, however, our data did not support our previous hypothesis, and the CLA-supplemented diet showed a similar effect on the reduction of joint inflammation as observed with the other modified diets. The fact that CLA-supplementation reduces the production of proinflammatory cytokines induced by

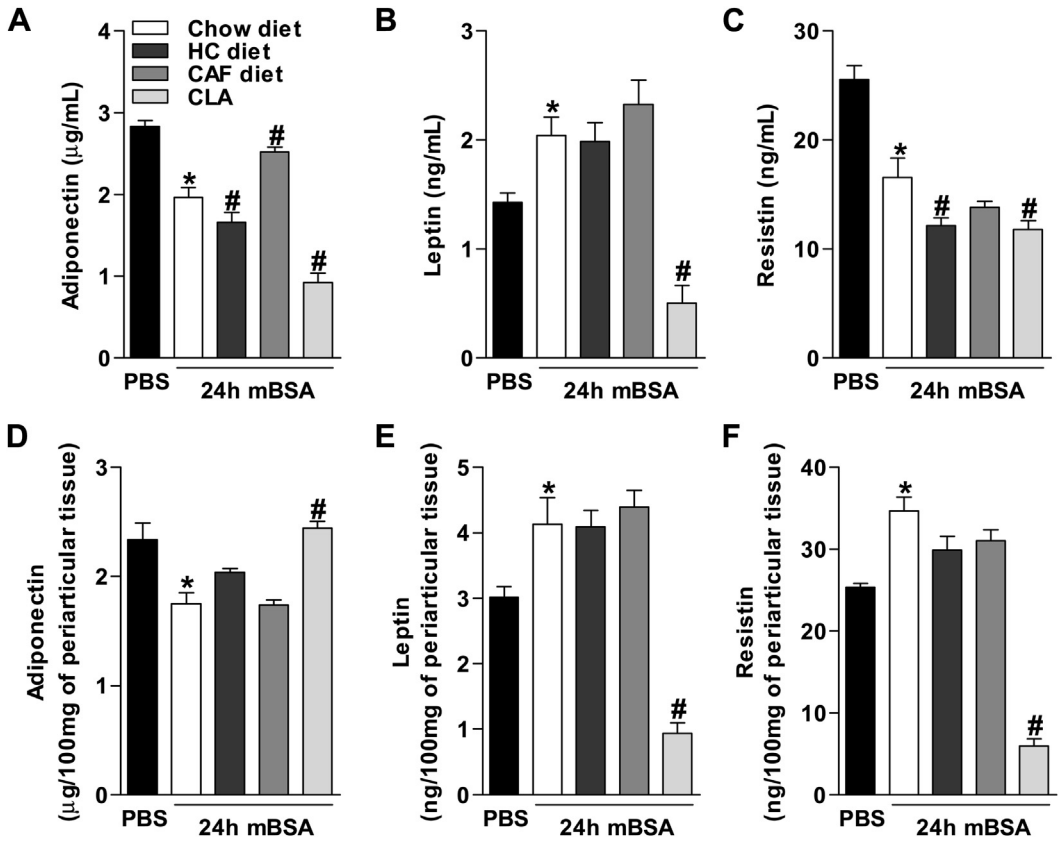

Fig. 6. Systemic and periarticular tissue alteration of adipocytokines at 24 h after antigen challenge. (A) Adiponectin, (B) resistin and (C) leptin levels in the serum, (D) Adiponectin, (E) resistin, and (F) leptin levels in the periarticular tissue of mice fed chow, a highly refined diet supplemented with carbohydrate (HC), cafeteria (CAF) diet, and conjugated linoleic acid (CLA)-supplemented diet at 24 h after antigen challenge in the joint. Bars represent the mean ± SEM ($n = 7–8$). *$P < 0.05$ versus PBS; #$P < 0.05$ versus chow diet mBSA group.

lipopolysaccharide has already been described [27]. Therefore, we suggest that alteration of the adipose tissue volume does not necessarily have an influence on the acute inflammatory response.

Neutrophils are the main cells recruited to the inflamed tissue; they are responsible for the tissue damage in this acute model of AIA [14,28]. We demonstrated that the HC diet decreases IL-1β levels and alters the activation of signaling molecules in periarticular tissue toward a reduced inflammatory condition. Extracellular signal–regulated kinase (ERK) belongs to the mitogen-activated protein kinase (MAPK) family and its activation occurs via MAPK signaling. It has been shown that the activation of ERK1/2 through its phosphorylation inhibits cellular apoptosis, being as it is a prosurvival factor and contributes to the inflammatory response [29]. There was an increase of p-ERK1/2 in the joints of the chow diet group mainly at the peak of inflammation (24 h), but it was lower in mice on the HC diet. Furthermore, the phosphorylated inhibitor of kappa B-alpha (IκB-α) was lower in mice on the HC diet 24 h after challenge with mBSA compared with the chow fed mice. The phosphorylation of IκB-α leads to activation of NF-κB, a molecule that contributes to the synthesis of several inflammatory markers as well as to cell survival [30], as observed in the inflamed joints of mice fed the chow diet. Interestingly, AnxA1, a molecule that has important antiinflammatory properties and contributes to the resolution of inflammation, was reduced in its intact form in AIA mice on the chow diet and consequently increased in its cleaved form. High intact AnxA1 levels are present in the cytoplasm of neutrophils, monocytes, and macrophages. Intact AnxA1 promotes neutrophil detachment and apoptosis and the phagocytosis of apoptotic neutrophils by macrophages [31]. However, mice fed the HC diet maintained relevant levels of intact AnxA1 and no increase in cleavage after antigen challenge. Moreover, the decreased number of neutrophils in the joints of the HC diet group is associated with inhibition of NF-κB and MAPK signaling pathways and the maintenance of intact AnxiA1, which could influence the resolution of inflammation.

In obesity, low-grade inflammation culminates in the abnormal production of cytokines by adipose tissue [10]. Adipocytokines act in the regulation of energy homeostasis, including self-regulation of the growth and development of adipocytes [10]. In obesity, the variation in the levels of adipocytokines may influence fat mass and insulin resistance [32]. Interestingly, the chow diet group showed alterations in adipocytokine levels similar to those of the AIA mice fed the HC and CAF diets. A similar profile was seen in hypertrophied adipocytes. Leptin is highly associated with the number of adipocytes [33]. Indeed, there was a direct correlation between leptin levels and adipocyte area in the knee. Because the adipose tissue is the main source of these adipocytokines [34], we consider that the local increase in adipocyte size could contribute to changes in the level of adipocytokines even in a state of acute inflammation. It was already demonstrated that CLA supplementation reduces the production of adipocytokines [35], as observed in our data. However, in a comparison with the other AIA groups, adiponectin levels in the knees of only the

CLA-supplemented group presented levels similar to those in nonarthritic mice. In chronic arthritis, adiponectin is considered a marker of progression and exacerbation of disease [36]. Thus, we believe that adiponectin, which generally is regarded as an antiinflammatory cytokine in acute inflammatory responses [37], also contributed to reduced inflammation in the knees of the CLA-supplemented mice. However, there is still a lack of studies exploring the direct contribution of adipocytes to the production of adipocytokines in the locally inflamed joint, as observed here in the case of infrapatellar adipocytes. Therefore, we suggest that acute joint inflammation contributes to adipocytokine production from the infrapatellar knee, which is mainly related to adipocyte size and could influence the inflammatory response.

We have shown that there were fewer neutrophils in the knee cavities of mice fed the HC and CAF diets than in those of control AIA mice, a difference related to increased adipose tissue. However, mice fed the CLA-supplemented diet, which had a reduction in adipose tissue, showed the same response. All of these diets are associated with systemic metabolic alterations [6,13,19]. It has been shown that metabolic changes may be involved in the reduction of neutrophil function, as observed in diabetic states [38]. Neutrophils isolated from healthy humans in the presence of different glucose concentrations showed abnormalities in neutrophil chemotaxis, phagocytosis, and microbicidal mechanisms [39]. In addition, hyperglycemia in vitro reduces the oxidative metabolism of isolated neutrophils [40]. Finally, diet-induced remodeling of adipose tissue as well as metabolic alterations may also influence the function of neutrophils by reducing their chemotaxis and activity.

Strengths and limitations

Obesity, a disease that is affecting more and more individuals all over the world, is related mainly to the uncontrolled consumption of diets rich in saturated fatty acids and refined carbohydrates as well as to a sedentary lifestyle [41]. The influence of high adiposity is important not only with regard to related comorbidities but also in terms of inflammatory diseases wherein obesity is a secondary disorder [21–24]. Our findings shed additional light on the acute inflammatory response, indicating that the quality of food can determine the profile of the inflammatory response.

Based on our data, we hypothesize that in the context of metabolic alterations caused by obesity or lipodystrophy, cell recruitment is impaired, thus attenuating the joint inflammatory response. This hypothesis still needs to be confirmed by experiments using a mechanistic approach. It would be interesting to evaluate the migration and function of neutrophils from mice treated with these obesogenic and lipodystrophic diets. However, the use of 3 different diets that alter the metabolism, even leading to changes in adipose tissue mass, shows that our hypothesis can be considered reasonable.

Conclusions

The present study shows that neutrophil influx into the knee joint was attenuated in mice fed with the HC, CAF, and CLA-supplemented diets after antigen induction. Moreover, the adipocyte area of the infrapatellar knee was influenced by an acute inflammatory response that can change the production of adipocytokines in the knee. We suggest that the remodeling of adipose tissue arising from a distinct diet composition is not directly related to the attenuated inflammatory response in acute arthritis; instead, we attribute this to metabolic alterations induced by obesity or lipodystrophy.

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Conflicts of Interest: None.

Authorship: MCO, LPS, MMT, FAA and AVMF formulated the research questions and designed the study. MCO, ALMS, LPT, and DFR conducted the experiments and analyzed the data. MCO, FAJL, LPS, MMT, FAA, and AVMF interpreted the data and wrote the manuscript. All of the authors read and approved the final manuscript.

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Chapter 4

Oral administration of bovine milk derived extracellular vesicles attenuates arthritis in two mouse models

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RESEARCH ARTICLE

Oral administration of bovine milk derived extracellular vesicles attenuates arthritis in two mouse models

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Scope: This study shows the effect of bovine milk derived extracellular vesicles (BMEVs) on spontaneous polyarthritis in IL-1Ra-deficient mice and collagen-induced arthritis.
Methods and results: BMEVs were isolated from semi-skimmed milk by ultracentrifugation and the particle size was around 100 nm by dynamic light scattering and electron microscopy. BMEVs expressed exosome marker CD63, immunoregulatory microRNA's (miR-30a, -223, -92a), and milk-specific beta-casein and beta-lactoglobulin mRNA. In vitro, PKH-67-labeled BMEVs were taken up by RAW264.7, splenocytes, and intestinal cells as determined by flow cytometry and confocal microscopy. IL-1Ra$^{-/-}$ mice received BMEVs by daily oral gavage starting at wk 5 till 15 after birth and collagen-induced arthritis mice via their drinking water starting 1 wk before immunization till day 40. Macroscopically, BMEV treatment delayed the onset of arthritis and histology showed diminished cartilage pathology and bone marrow inflammation in both models. BMEV treatment also reduced the serum levels of MCP-1 and IL-6 and their production by splenic cells. BMEV treatment diminished the anticollagen IgG2a levels, which was accompanied by reduced splenic Th1 (Tbet) and Th17 (RORγT) mRNA.
Conclusion: This is the first report that oral delivery of BMEVs ameliorates experimental arthritis and this warrants further research to determine whether this beneficial effect can be seen in rheumatoid arthritis patients.

Keywords:
Exosomes / Immunity / MicroRNA / Milk / Rheumatoid arthritis

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1 Introduction

Development of rheumatoid arthritis (RA) is associated with genetic and environmental factors and several studies show a connection with lifestyle and dietary factors. The "Western diet" (high of sugar, salt, and fatty acids) has been implicated to contribute to the development of RA possibly by modulating T-cell responses [1]. Cow milk and dairy products are part of the current Western diet. RA patients are advised to drink milk to promote bone mineral density and counteract osteoporosis, a serious side effect of long-term anti-inflammatory glucocorticoid treatment. Bovine milk is a source of lactose, proteins, fat, calcium, and vitamins [2] and intake may improve inflammatory and oxidative stress markers [3]. A prospective study [4] shows that frequent and high milk consumption (≥7 glasses/wk) by women is associated with reduced progression of osteoarthritis. This was linked to the known beneficial effect of calcium and vitamin-D on bone. In contrast, a retrospective study of the Swedish population [5] shows that high milk consumption (≥3 glasses/day) is associated with more hip fractures in women. The authors explanation is that milk is the main dietary source of D-galactose, a sugar that is deleterious to health by promoting ageing [6].

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Abbreviations: **APC**, antigen-presenting cell; **βLG**, beta-lactoglobulin; **BMEVs**, bovine milk derived extracellular vesicles; **CIA**, collagen-induced arthritis; **DLS**, dynamic light scattering; **miRNA**, microRNA; **RA**, rheumatoid arthritis; **TGF-β**, transforming growth factor beta

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Evidence linking milk consumption to RA development is not available. Interestingly, RA patients have strikingly higher antibody levels against food components in their gut and blood, including IgM against casein [7]. Casein (α, β, κ) is the major milk protein (80% of total protein content) followed by β-lactoglobulin (βLG; 10%) and α-lactalbumin (2%). The IgM levels against the latter two proteins were also significantly increased in the gut but not in serum of RA patients. That milk antigens promote RA is corroborated by studies showing that milk intake leads to synovitis in susceptible rabbits [8].

The discovery of food-derived microRNA (miRNA) in the circulation and even human breast milk suggest that miRNAs could also be a functional food component [9]. Still there is considerable doubt about the correctness of these studies, so whether food miRNA are taken up by our body is debatable [10]. MiRNA are small (~22 nucleotide) noncoding RNAs that regulate gene expression at the posttranscriptional level [11]. When shed by the cell as an extracellular vesicle (EV), the miRNA might be more protected against the hostile environment in the gut. EVs, including exosomes and microvesicles, are membrane vesicles secreted by a variety of cells and are heterogeneous in size, ranging from 30 to 1000 nm in diameter [12]. Although EVs were previously considered to be cellular waste products, they are now believed to be an important mediator in facilitating intercellular communication [13]. Recent studies also revealed that EVs are involved in the genetic exchange of RNA and miRNAs between cells [14]. EVs have been identified in a number of biological fluids such as blood, saliva, and urine [15, 16]. In serum and saliva it is has been shown that the majority of miRNAs are present in exosomes [17].

Exosomes have also been identified in both human breast milk and bovine colostrums [18, 19]. It has been suggested that these breast milk-derived EVs are taken up systemically by the milk recipient, where they can play a role in the development of the infants immune system [20]. Drinking cow milk may expose humans to immunoregulatory miRNA that are encapsulated in bovine milk derived extracellular vesicles (BMEVs). Furthermore, food-derived exosomes are able to regulate endogenous gene expression in animals [21].

In this study, we investigate whether BMEVs themselves do have an effect on the development of arthritis. For that, mice received BMEVs in their drinking water or via oral gavage and the development of polyarthritis in IL-1Ra-deficient mice and collagen-induced arthritis (CIA) in DBA1/J mice was studied. BMEVs delayed the onset of disease in both models and diminished cartilage pathology and bone marrow inflammation.

2 Materials and methods

2.1 Milk samples and EVs preparation

A differential centrifugation protocol (3000 × *g* for 15 min, 12 000 × *g* for 1 h, 35 000 × *g* for 1 h, 70 000 × *g* for 1 h) was used to remove fat globules, casein aggregates, and other debri from the semi-skimmed pasteurized (72°C for 15 sec) cow milk [22]. Next, the supernatant was filtered through Whatman papers nr1 and nr50 followed by a 0.2 μm syringe filter. EVs were pelleted by ultracentrifugation (110 000 × *g* for 1.5 h at 4°C), dissolved in PBS (o/n at 4°C) on a tube rotary system, thereafter centrifuged (200 × *g* for 10 min at 4°C) to remove aggregates and 0.2 μm filter sterilized. The protein content was measured with a Micro-BCA kit (Thermo Scientific, Pierce, Rockford, USA). The endotoxin level in the BMEV batch used in all experiments was below the detection of <01000 EU/mL as measured with the LAL assay (Endosafe, Charles River).

2.2 Dynamic light scattering (DLS)

DLS and zeta potential determinations were performed with a Zetasizer nanoseries instrument (Malvern Nano-Zetasizer, λ = 532 nm laser wavelength) [23]. The exosome size data refers to the scattering intensity distribution (*z* average). For calibration 100 nm Latex beads (Agilent Technology, Santa Clara, CA, USA) were used.

2.2 Total RNA isolation

Messenger RNA and miRNAs were isolated from BMEVs as previously described [22]. Briefly, TRI reagent (Invitrogen, Carlsbad, CA, USA) and chloroform were added to the BMEV pellet and after centrifugation isopropanol was added to the supernatant and incubated overnight. Thereafter centrifuged and the pellet was washed twice with 75% ethanol and dissolved in RNase-free water.

2.3 Quantitative real-time PCR

Isolated RNA was treated with DNaseI (Invitrogen) to remove DNA contamination. Synthesis of cDNA was accomplished by reverse transcription PCR using an oligo (dT) primer and Moloney murine leukemia virus reverse transcriptase (Applied Biosystems Inc., Foster City, CA). Quantitative real-time PCR was performed using SYBER-Green on a Step-One according to the manufacturer's instructions (Applied Biosystems Inc.) with primer sets described in Table 1. For miRNA detection, cDNA was generated from DNaseI-treated RNA, using a QuantiMir RT Kit (System Biosciences, Mountain View, CA, USA) according to the manufacturer's instructions. PCR products were amplified using specific primers for miRNAs (S1) and the universal QuantiMir reverse primer. PCR product was visualized on a 3% agarose ethidium–bromide gel.

2.4 Transmission electron microscopy

BMEVs were prepared on a 30% sucrose cushion (110 000 × *g* for 70 min), and pelleted by an additional ultracentrifugation

Table 1. Bovine mRNA and miRNA primer sequences

Target	Accession number	Sequence (miRBase)
GAPDH	BC102589	Fw: GGGTCATCATCTCTGCACCT Rv: ATCCACAGTCTTCTGGGTGG
Elongation factor-1alpha	AB060107	Fw: ATTTGTGCCAATTTCTGGCT Rv: AGACATCCTGGAGAGGCAAA
Beta-casein	NM_181008	Fw: GGATTTCAAAGTGAATGCCC Rv: TGATGCAAGGATTGAAAAGTTG
Beta-lactoglobulin	NM_173929	Fw: CGATGCCTTGAATGAGAACA Rv: TTTGTCGAATTTCTCCAGGG
bta-miR-30a	MI0005054	TGTAAACATCCTCGACTGGAAGC
bta-miR-92a	MI0009905	TATTGCACTTCTGGGCCGGTCT
bta-miR-223	MI0009782	TGTCAGTTTGTCAAATACCCCA

step [24]. EVs in deionized water were placed on a nickel grids and allowed to dry for 45 min. Grids were washed by transferring them onto several drops of deionized water and were incubated for 1 h at RT with mouse anti-bovine CD63 antibody (1:50) or mouse anti-bovine IgG as a control. Grids were washed again and were incubated with gold labeled goat anti-mouse antibodies (1:50) for 1 h at RT. Unbound antibodies were washed away and the grids were dried before analysis on a Jeol JEM1400 Transmission Electron Microscope (Jeol, The Netherlands).

2.5 Cellular vesicle uptake

BMEVs were stained with PKH67 Green Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Sigma-Aldrich, St. Louis, MO) and free PKH67 label was removed by 300 kDa Vivaspin filters (Sartorius Stedim Biotech GmbH, Goettingen, Germany). PKH67-labeled BMEVs were incubated with RAW264.7 at indicated times at either 4°C or 37°C. After incubation, cells were washed twice, fixed with 4% paraformaldehyde and stained with 4,'6'-diaminido-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). Cellular uptake of BMEVs was determined using fluorescent and confocal microscopy (Leica microscopy, Rijswijk, The Netherlands) or FACS analysis (Becton Dickinson, Mountain View, CA). For confocal microscopy, a F4/80 antibody (AbD Serotec) was used for membrane staining and DRAQ5 (Cell Signaling Technology) to detect nuclei.

One end of the isolated mouse ileum was closed with suture thread, then 100 μL of PKH-67-labeled BMEVs was pipetted into the lumen and the other end closed. After culturing with 5% CO_2 for 24 h in RPMI-1640 (Life Technologies) with 5%FCS and penicillin/streptomycin, both ends were opened and flushed three times with PBS to get rid of unbound BMEVs. BMEV uptake was measured by the IVIS Lumina (Caliper Life Science). As a control-labeled BMEVs were incubated at 4°C and for autofluorescence correction the ileum explants were cultured with unlabeled BMEVs.

2.6 Splenic antigen-presenting cells (APC) and T-cell isolation and stimulation ex vivo

Spleens were removed from 12-wk-old male C57Bl/6 mice (Janvier-Elevage, Saint Berthevin, France), mashed, passed through a 70 μm strainer and erythrocytes were removed by osmotic shock. The cell suspension was resuspended in 5% exosome-free fetal bovine serum containing RPMI1640 medium (Invitrogen, Carlsbad, CA) and incubated at 37°C in 5% CO_2 for 1 h to obtain the adherent fraction that mainly consisted out of APC. The adherent cells were incubated for 24–48–72 h with 0–20–200 μg/mL BMEVs. Thereafter, APCs were stimulated for 6 h with LPS (10 ng/mL) and cytokine production was analyzed by Luminex multianalyte technology (Bio-Rad, Hercules, CA).

For Treg differentiation, CD4+CD62L+ splenic T cells were isolated from the nonadherent fraction by MACS (Miltenyi Biotech, Leiden, The Netherlands) and cultured in the presence of anti-CD3 (5 μg/mL) and anti-CD28 (2.5 μg/mL) with IL-2 (160 IU/mL; Bioconnect, Huissen, The Netherlands) and either EVs (400 μg/mL) or transforming growth factor beta (TGF-β, 2.5 ng/mL) for 4 days. MAB1835 (5 μg/mL) was used to determine TGF-β dependency of EV-induced Treg differentiation. To assess Treg differentiation, cells were restimulated for 4 h with phorbol myristate acetate (50 ng/mL) and ionomycin (1 μg/mL) (Sigma-Aldrich), thereafter RNA was isolated to determine expression of Foxp3 and CD25.

2.7 Arthritis models

IL-1Ra$^{-/-}$ mice on the BALB/c background spontaneously developed a polyarticular arthritis between 5 and 8 wk of age [25]. Mice received BMEVs daily by oral gavages started before onset of arthritis at week 5 of age. The CIA model was induced in male 10- to 12-wk-old DBA/1J mice (Janvier, Elavage, France) by immunization with bovine collagen type II as described before [26]. BMEVs were added to the drinking water 7 days before the start of the immunization. Clinical

severity of arthritis (arthritis score) was scored macroscopically by two independent observers in a blinded fashion on a scale of 0–2 for each paw (0, no changes; 0.25, 1–2 toes red or swollen; 0.5, 3–5 toes red or swollen; 0.5, swollen ankle; 0.5, swollen footpad; 0.5, severe swelling and ankylosis (redness, excessive edema, and deformation). All mice were housed in filter-top cages, and a standard diet and water were provided ad libitum. The in vivo studies complied with national legislation and were approved by the local authorities on the care and use of animals (DEC-nr:2014-083).

2.8 Histological assessment

Murine ankle joints were isolated, fixed in 4% phosphate buffered formalin (pH 7.4) and decalcified in 5% formic acid. Ankle joints were dehydrated with an automated tissue-processing apparatus (LeicaASP300, Rijswijk, The Netherlands) and embedded in paraffin. Ankle joint sections of 7 μm thickness were stained with Safranin O (BDH Chemicals, Poole, UK) and counterstained with fast green (BHD Chemicals). Histopathological changes were scored in the calcaneus region of the ankle joint by two independent observers in a blinded fashion. Cartilage depletion, defined as the loss of proteoglycan content, was scored (range 0–3) per region. Infiltration of cells was scored ranging between 0 and 3 (0, no cells; 1, mild cellularity; 2, moderate cellularity; 3, maximal cellularity). Cartilage erosion was graded (range 0–3), from no damage to compete loss of articular cartilage. Bone marrow cellularity was scored ranging between 0 and 3 (0, few myeloid cells and most of it are large fat cells; 1, mild cellularity; 2, moderate cellularity; 3, maximal cellularity).

2.9 Determination of specific IgG serum titers against type II collagen in serum

The serum IgG1, IgG2a, and total IgG antibody titers against bovine type II collagen were determined by ELISA. Briefly, 96-well plates were coated with 10 μg of bovine type II collagen and nonspecific binding sites were blocked. Serial dilutions of mouse sera were added, followed by incubation with isotype-specific goat anti-mouse peroxidase diluted 1:2.000 in PBS (Southern Biotechnology, Birmingham, AL) and substrate 5-aminosalicylic acid (Sigma, Poole, UK). Plates were measured at an optical density of 450 nm.

2.10 Cytokine measurements

TNF-α and MCP-1 levels were determined using the Luminex multianalyte technology, using the BioPlex system in combination with BioPlex Mouse Cytokine Assays (Bio-Rad, Hercules, CA). Cytokines were measured in 50 μL washout medium and sensitivity was <3 pg/mL.

2.11 Statistical analysis

All data are expressed as the mean ± SD or mean ± SEM. Data were compared using Mann–Whitney U test or one-way ANOVA. Values of $p < 0.05$ were considered to indicate statistical significance. All statistical analyses were performed using GraphPad Prism 5.01 (GraphPad Software, La Jolla, CA).

3 Results

3.1 Characterization of BMEVs

The mean diameter of BMEVs isolated from commercially available semi-skimmed cow milk was around 100 nm as measured by DLS (Fig. 1A). Nanoparticle tracking analysis showed a size distribution between 100–150 nm with a mean diameter of 125 nm [22]. Analysis by asymmetric field flow fractionation showed three peaks with an average calculated radius of 20, 60, and 107 nm (data not shown). Proteomic analysis of the BMEVs identified 64 proteins some transmembrane proteins (CD36, CD59, CD81), cytosolic proteins (Rab-1b, Rab-11a), intracellular structural proteins (actin, myosin-XIX), and precursor of secreted milk proteins (casein, α-lactalbumin, βLG, osteopontin) (Supporting Information 1). We identified by RT-qPCR a number of immune-related miRNAs (miR-30a, -223, -92a) (Fig. 1B) and mRNAs of milk-specific proteins (α-casein, βLG, and elongation factor-1α) (Fig. 1C). On transmission electron microscopy, the BMEVs had a diameter between 100 and 150 nm, a morphology similar to exosomes, and gold immunolabeling (particle size 10 nm) demonstrated presence of CD63, a tetraspanin, and known membrane marker of exosomes (Fig. 1D). Our isolated milk particles fulfilled all the international criteria of being EVs [27].

3.2 Cellular uptake of BMEVs

RAW264.7 macrophages showed a fast uptake, within 1–3 hours, of PKH-67-labeled BMEVs as examined using fluorescent microscopy and flow cytometric analysis (Fig. 2A and B, respectively). Biophotonic imaging of ileum explants showed clear uptake of BMEVs by this tissue after 24 h incubation at 37°C (Fig. 2C and D). Confocal microscopy showed the cytoplasmic location of BMEVs that are taken up by splenocytes and ileum cells (Fig. 2E). In all cases, uptake was markedly impaired when incubated at 4°C, excluding passive uptake, nonspecific adherence, or uptake of free PKH-67 dye. Used BMEVs concentrations showed no toxicity on RAW264.7 cells nor splenocytes as measured by XTT viability assay (data not shown).

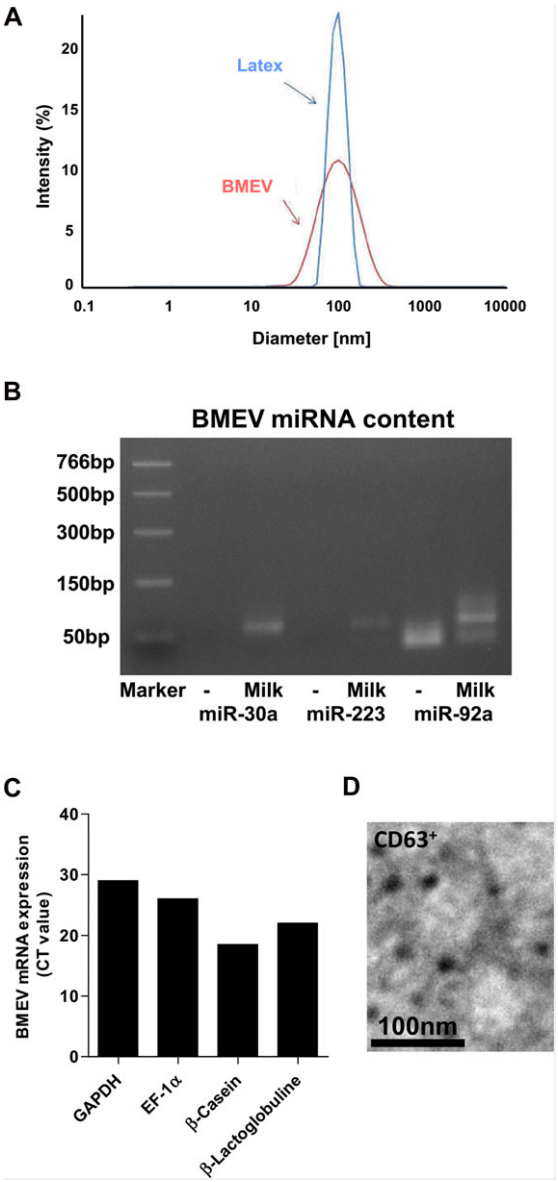

Figure 1. Characterization of BMEVs. Particle size distribution of isolated BMEVs or standard latex beads (100 nm) determined by DLS. (A) Detection of immunoregulatory miRNAs (miR-30a, miR-92a, miR-223) present in BMEVs by RT-PCR. (B) Detection of bovine-specific mRNA β-casein, βLG and elongation factor-1α in BMEVs by RT-qPCR. (C) Immunostaining of BMEVs with gold-labeled antibodies for CD63 detected by electron microscopy. (D) Gold particles were 10 nm of size.

3.3 Immunoregulatory properties of BMEVs

Adherent splenocytes from naïve mice were preincubated for 24, 48, and 72 hours with two different concentrations of BMEVs (20 and 200 μg/mL) and then stimulated with LPS (10 ng/mL) for 6 hours. Cytokine levels without LPS stimulation were 0.7 pg/mL TNF-α and no detectable MCP-1 in the group without BMEVs and 200 μg/mL BMEVs, 1.4 pg/mL TNF-α, and 8.1 pg/mL MCP-1 in the 20 μg/mL BMEV group. BMEV treatment clearly reduced the LPS-induced TNF-α and MCP-1 production at all time points studied (Fig. 3A and B). Next the differentiation of naïve splenic CD4+CD62L+ T cells into regulatory T-cells (Tregs) was studied. As expected TGF-β (2.5 ng/mL) increased the expression of Treg-specific Foxp3 and CD25 mRNA expression (Fig. 3C). Incubation with BMEVs (400 μg/mL) also enhanced the differentiation into Tregs and this could be blocked by the addition of anti-TGF-β1,2,3 antibodies (5 μg/mL) (Fig. 3C). This shows that BMEVs can be anti-inflammatory and may suppress the adaptive immune response.

3.4 Oral administration of BMEVs ameliorates experimental arthritis

IL-1Ra$^{-/-}$ mice at week 5 of age received a daily dosage of BMEVs by oral gavage until the end of the experiment. Mice receiving a dosage of 4×10^6 BMEVs (low, approximately 170 microgram/mL) showed no differences in onset of disease and macroscopic score while mice receiving the higher dose 28×10^6 (high, approximately 1200 microgram/mL) showed a considerable delay in disease onset and macroscopically reduction in swelling of the ankle joints (Fig. 4A and B). Histological analysis at week 9 of treatment showed significantly reduced cartilage depletion and bone marrow cellularity in the high BMEV treatment group while reduction in joint inflammation (infiltrate and exudate) did not reach statistical significance (Fig. 4C). Isolated adherent splenocytes from arthritis IL-1Ra$^{-/-}$ mice expressed the chemokines IL-6, KC, and MCP-1. Splenocytes from the low BMEV treatment group showed a trend toward reduced IL-6, KC, and MCP-1 expression that reached significance in the high BMEV treatment group (Fig. 4E). To analyze the effect on adaptive immunity, isolated intestinal T cells were isolated and both BMEV treatment groups resulted in enhanced T-bet expression (transcription factor of Th1). Only the high BMEV treatment group showed a trend to enhanced expression of GATA-3 (Th2), IL-17 (Th17) and significantly higher Foxp3 (Treg) expression (Fig. 4F).

Next, we studied the effect of BMEVs on CIA in DBA/1J mice, a strain sensitive to environmental stress [28]. For that, BMEVs were not provided by oral gavage but was added to their drinking water in two different concentrations (4.7×10^6/mL (low, approximately 38 microgram/mL) or 14.3×10^6/mL (high, approximately 115 microgram/mL)) started 1 wk before the immunization till the end of the experiment and was refreshed every 2 days. Clinical development of arthritis was delayed in both BMEV treatment groups but only in the high BMEV treatment group mice remained behind in arthritis development (incidence and severity) (Fig. 5A and B). At day 40 after immunization, histology of the ankle joints showed reduced joint inflammation, cartilage depletion, and bone-marrow cellularity in a dose-dependent manner although this did not reach significance (Fig. 5C and

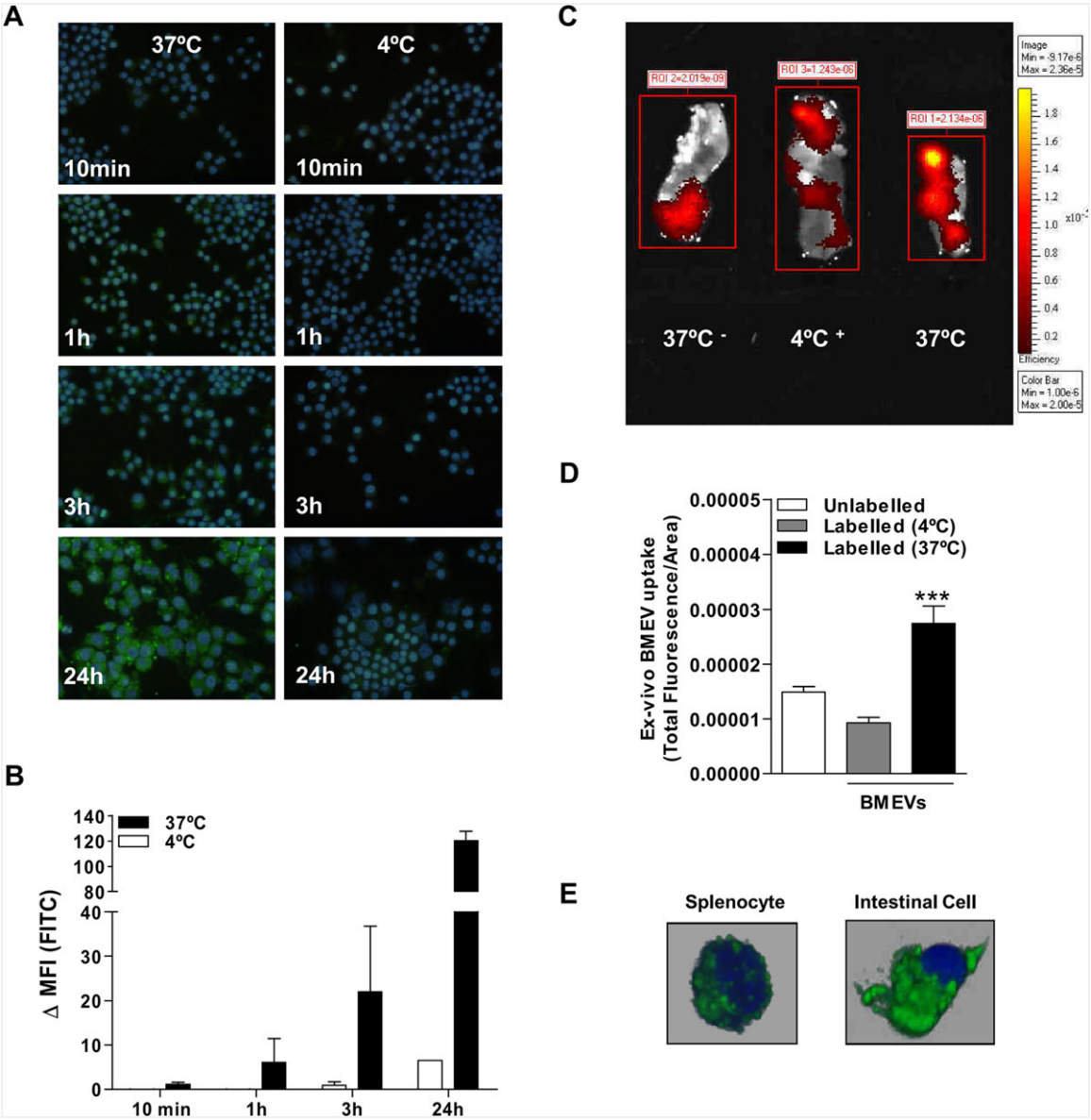

Figure 2. Cellular uptake of BMEVs in murine cells. Time-dependent uptake of PKH67-labeled BMEVs (green) by RAW264.7 macrophages incubated at 37°C but not at 4°C. (A) Representative microscopic images for three separate experiments. Flow cytometric analysis (FITC wavelength) showed enhanced cellular uptake of PKH67-labeled BMEVs by RAW264.7 macrophages. (B) The mean fluorescence intensity (MFI) of two separate experiments (performed in duplicate) was averaged. Isolated murine intestines were incubated with PKH67-labeled BMEVs in their lumen for 24 h at 37°C or 4°C to control for active uptake. Unlabeled BMEVs incubated at 37°C were used to detect background signal. (C) Photonic imaging (IVIS Lumina) showed enhanced fluorescent signal intensity in ileum explants incubated with PKH67-labeled BMEVs at 37°C. (D) Confocal microscopy confirmed the intracellular localization of phagocytozed BMEVs (green) by isolated splenocytes and intestinal cells. (E) Nucleus was stained with DRAQ5 (Blue). Error bars represent mean ± SD of triplicates. Statistically significant differences were determined by Mann–Whitney test, $^{***}p < 0.001$.

D). In line with this, only the high BMEV treatment group showed reduced circulating levels of MCP-1 and IL-6 (Fig. 2A). Unexpectedly, both BMEV treatment groups showed markedly reduced mRNA expression of T-bet (Th1) and ROR-γT (Th17) expression in primary splenocytes while no effect was seen on the anti-inflammatory GATA-3 (Th2) and Foxp3 (Tregs) cells (Fig. 6B). Also the circulating levels of anticollagen type II antibodies of total IgG and isotype IgG2a (a marker of Th1) were reduced in both BMEV treatment groups (Fig. 6C and D). The levels of anticollagen type II isotype IgG1 (marker of Th2) was not changed by BMEV treatment (Fig. 6E).

4 Discussion

Commercially available cow milk contains EVs with characteristics of exosomes. Oral delivery of these BMEVs caused a profound delay of disease development in two T-cell-dependent chronic and destructive murine arthritis models with marked protection of cartilage pathology and bone marrow inflammation in the IL-1Ra$^{-/-}$ model and a trend in the CIA model. BMEV treatment had a marked inhibitory effect on the innate immunity as illustrated by reduced expression of MCP-1 (CCL2), KC, and IL-6. The effect of BMEV treatment on adaptive immunity is enhanced in the gut (IL-1Ra-/- model) and reduced in the spleen (CIA model). This is the first demonstration of a modulating effect of BMEVs on two models of polyarticular autoimmune arthritis via the natural route of oral uptake.

Commercial semi-skimmed milk contains a high amount of EVs ($\approx 5 \times 10^9$ particles ($= 40$ mg protein)/200 mL) and we and others have found that BMEVs can resist the harsh condition of the gut [22, 29]. Some investigators have speculated that exosomal miRNAs are transferred from the mother's milk to the infant via the digestive tract and reach the infant's circulation and organ systems [30]. Whether milk-EVs target the intestinal epithelium, or via a transcytosis process pass the intestinal epithelium and enter the body or stay in the digestive tract has to be determined. However, we could show that in vitro the small intestine and intestinal cells could take up BMEVs by an active process (Fig. 2).

We clearly showed that oral delivery of BMEVs delays the onset of disease in two murine arthritis models. This is in contrast to the finding by Hanglow and Welsh [31, 32] that 12 wk of cow milk feeding caused synovitis in susceptible rabbit strains. Cow milk contains many biological molecules and we identified BMEVs as stable particles that contain bioactive TGF-β and immunoregulatory miRNAs. The protective effect on arthritis could be due to TGF-β on BMEVs as we and others showed in vitro that TGF-β mediates both BMEV-induced regulatory T-cell differentiation and under inflammatory cytokine conditions Th17 cell differentiation [18, 22]. In the IL-1Ra knockout model we found that a high dose of BMEVs enhanced the Th1-related transcription factor T-bet and Treg-related Foxp3 in intestinal CD4$^+$ cells and simultaneously higher IL-17 expression. It has been described that human Foxp3$^+$ Tregs can differentiate into Th17 [33], and that RORγT$^+$ Foxp3$^+$ Tregs are suppressive in CIA [34]. This shows the plasticity of T cells and we only can conclude from our data that BMEVs do affect T-cell immunity in the gut either directly or indirectly via e.g. modulating the intestinal microbiome.

In the CIA model, we found at the end of the experiment that the expression of T-bet and RORγT in splenocytes were reduced in both BMEV groups. Whether this is an indirect effect of BMEVs due to delayed arthritis onset remains to be evaluated. The reduced circulating levels of anticollagen type II IgG2a but not IgG1 is in agreement with a lower Th1 activity

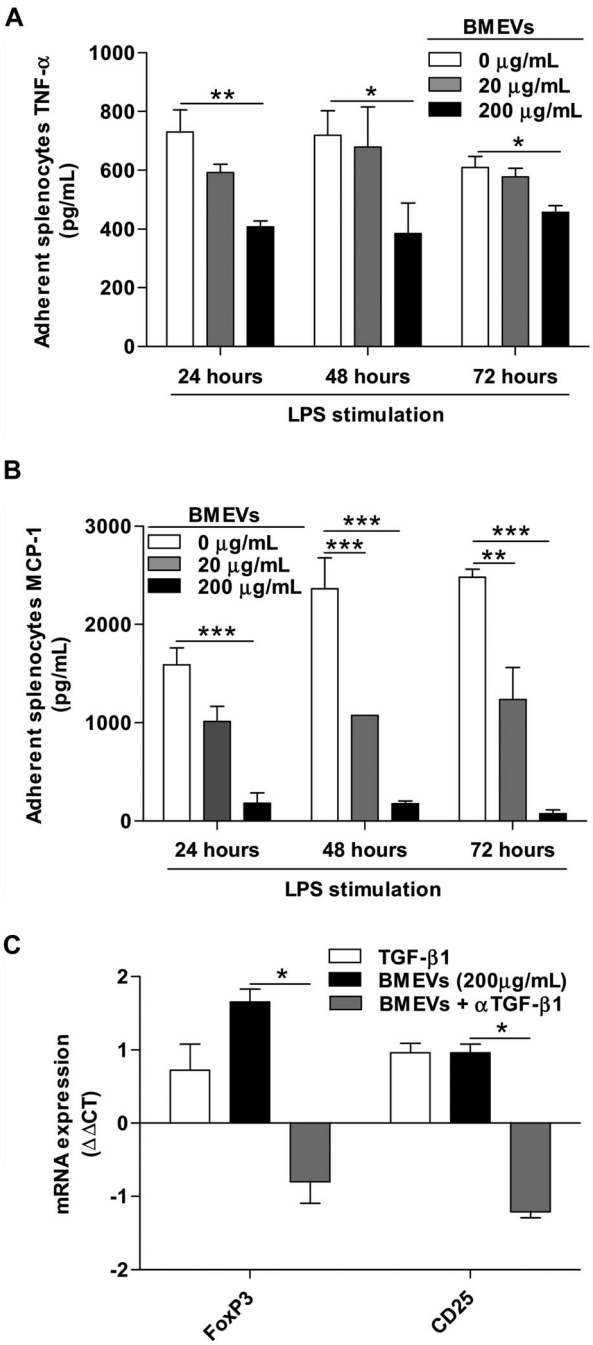

Figure 3. Functionality of BMEVs in vitro. Isolated adherent murine splenocytes were incubated 24, 48, and 72 h with BMEVs (20, 200 μg/mL) and thereafter stimulated with LPS (10 ng/mL) for 6 h. Production of (A) TNF-α and (B) MCP-1 were measured by multiplex bead array assay. Induction of Treg differentiation from isolated murine splenic CD4 + CD62L + T cells by TGF-β (1.25 ng/mL) or BMEVs (200 μg/mL) and measured by the increased expression of Foxp3 and CD25 mRNA. (C) Blocking TGF-β using an anti-TGF-β1,2,3 antibody (5 μg/mL) inhibited BMEV-induced Treg differentiation. Statistically significant differences were determined by Mann–Whitney test, $^*p < 0.01$, $^{**}p < 0.05$, $^{***}p < 0.001$. Error bars represent mean ± SD ($n = 4$).

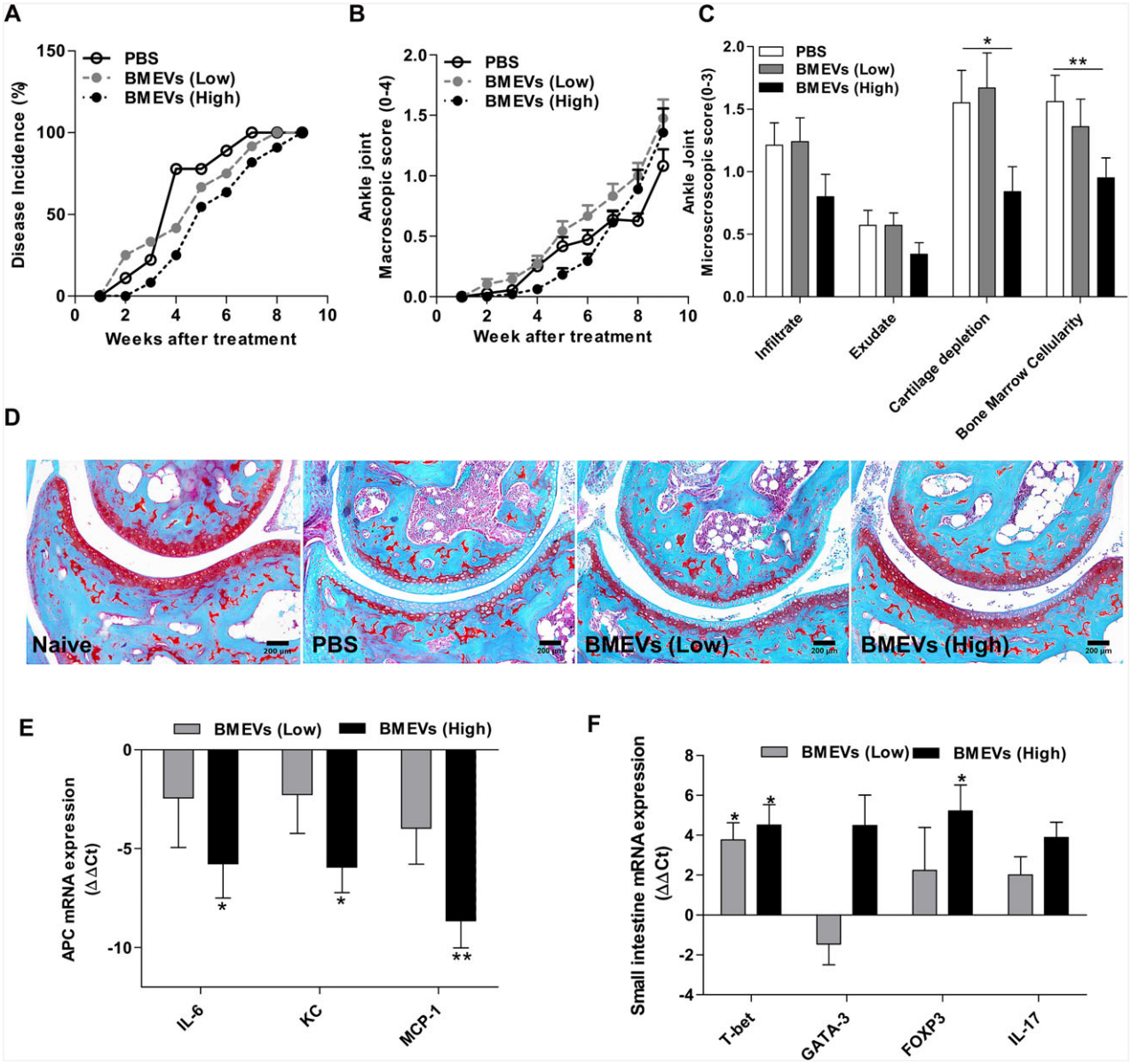

Figure 4. Functionality in vivo in IL-1Ra-/- mice. IL-1Ra$^{-/-}$ mice received BMEVs (4×10^6 (low) or 28×10^6 (high) particles) or PBS (vehicle) daily by oral gavage started at age wk 5. Onset of arthritis was scored at indicated time points. (A) Severity of arthritis in hind paws was monitored macroscopically for 9 wk. (B) Histological analysis of inflammation (infiltrate, exudates, cartilage depletion, and bone marrow cellularity) in the ankle joints. (C) Data are represented as mean ± SEM ($n = 12$) and statistical differences were calculated using one-way ANOVA test. $^{*}p < 0.05$, $^{**}p < 0.01$. Representative histology of ankle joints of each group. (D) IL-6, KC, and MCP-1 mRNA levels were quantified in isolated adherent splenocytes (APC) of the treated mice by RT-qPCR. (E) Bars represent the mean ± SEM ($n = 7$) of each group and statistical differences were calculated using Mann–Whitney *U* test. $^{*}p < 0.05$, $^{**}p < 0.01$. mRNA levels of transcription factors T-bet, GATA-3, and Foxp3 and IL-17 in isolated intestinal cells were detected by RT-qPCR. (F) Data are represented as mean ± SEM ($n = 3$) and statistical differences were calculated using Mann–Whitney *U* test. $^{*}p < 0.05$.

and unchanged Th2 in both experimental groups. Generally the reticuloendothelial system in the spleen, liver, and lung is found to be the primary target of nanosized particles including exosomes [35, 36]. The observed reduction of IL-6, KC (CXCL1), and MCP-1 expression in splenic APCs and lower circulating levels of MCP-1 and IL-6 after BMEV treatment in mice suggest that the BMEVs also did target the monocytes, macrophages, and dendritic cells after oral administration.

It has been described that BMEVs contain many immunoregulatory miRNA [30, 37, 38] such as miR-124a that directly targets MCP-1 mRNA [29, 39]. Other miRNAs (mir-21, mir-let7, mir-146a, mir-146b, mir-126, mir-155, mir-199a)

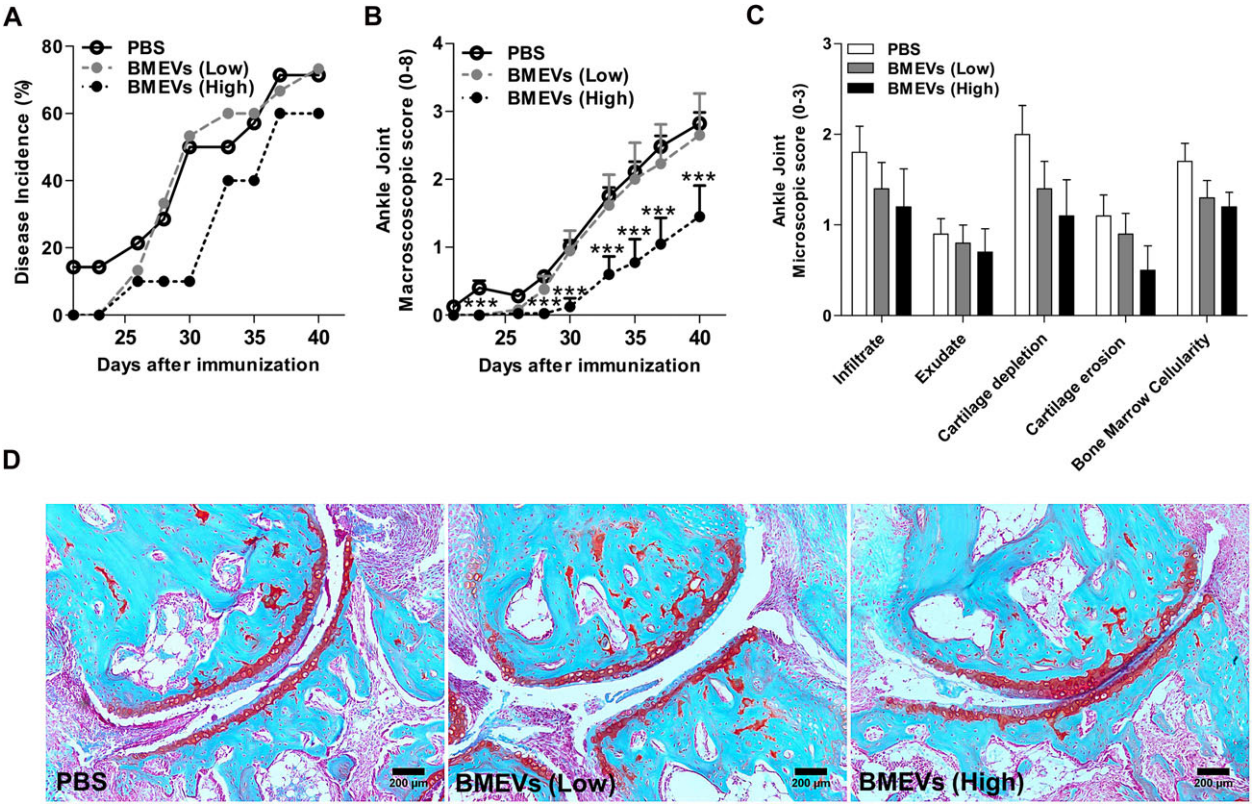

Figure 5. Functionality in vivo in CIA model. One week before immunization with collagen mice received BMEVs (4.7×10^6/mL (low) or 14.3×10^6/mL (high)) via their drinking water. Onset of arthritis was scored at indicated time points. (A) Appearance of arthritis in front and hind paws was monitored till day 40 after immunization. (B) Histological analysis of inflammation (infiltrate, exudates, cartilage depletion, cartilage erosion, and bone marrow cellularity) in the ankle joints. (C) Representative histology of the ankle joints in each group. (D) Data are represented as mean ± SEM ($n = 11$) and statistical differences were calculated using one-way ANOVA test. $^{***}p < 0.001$.

can target the TLR/IL-1 inflammatory pathway [40, 41]. Besides the miRNAs also some protein present in BMEVs exert an anti-inflammatory effect such as lactoferritin, apolipoprotein E, and TGF-β [42–44].

Another observed effect was the markedly diminished cellularity of the bone marrow in the BMEV groups. Enhanced bone marrow cellularity is also called osteitis and is characterized by infiltration of inflammatory cells (predominantly monocytes/macrophages, and smaller number of B cells), myelopoiesis, and edema. Several studies showed that osteitis is a much stronger predictor of bone destruction progression than synovitis [45]. In both adjuvant and CIA in rats, bone marrow IL-1 levels in conjunction with myelopoiesis rise before onset of arthritis and this correlates with progression of disease [46]. In RA patients, the bone marrow is a source of IL-6, IL-8 (in mouse called KC), and MCP1 [47, 48]. In the highest BMEV treatment groups, we found in both arthritis models a reduced expression of these cytokines and chemokines that may underpin the observed effect of BMEVs on bone marrow cellularity. Bone marrow is also the major site of antibody production and MCP-1 is also a major chemokine for B-cells. Therefore, the BMEV treatment reduced bone marrow osteitis and diminished anticollagen type II antibody titers in CIA mice could be inextricably linked.

We conclude that BMEVs exert a therapeutic effect on autoimmune arthritis models when delivered orally. The exact mechanism of protection by BMEVs remains, however, to be determined and as some observed effects were dose dependent the maximum effect of BMEVs might not have been reached in our experiments. Our study shows for the first time that BMEVs can exert a biological effect in vivo and this warrants further research to fully elucidate its therapeutic potential in the treatment of autoimmune and inflammatory diseases.

O.A., B.P., and F.L., participated in the design of the study. O.A., B.P., M.C., and M.B.B., contributed in the experimental methods. O.A., B.P., M.C., M.B.B., and M.G.B. performed data analysis. O.A., B.P., F.L., and M.C., wrote the manuscript. O.A., B.P., M.C., M.G.B., M.B.B., M.V., P.L., P.K., M.K., W.B., and F.L., contributed to discussions and approved the manuscript.

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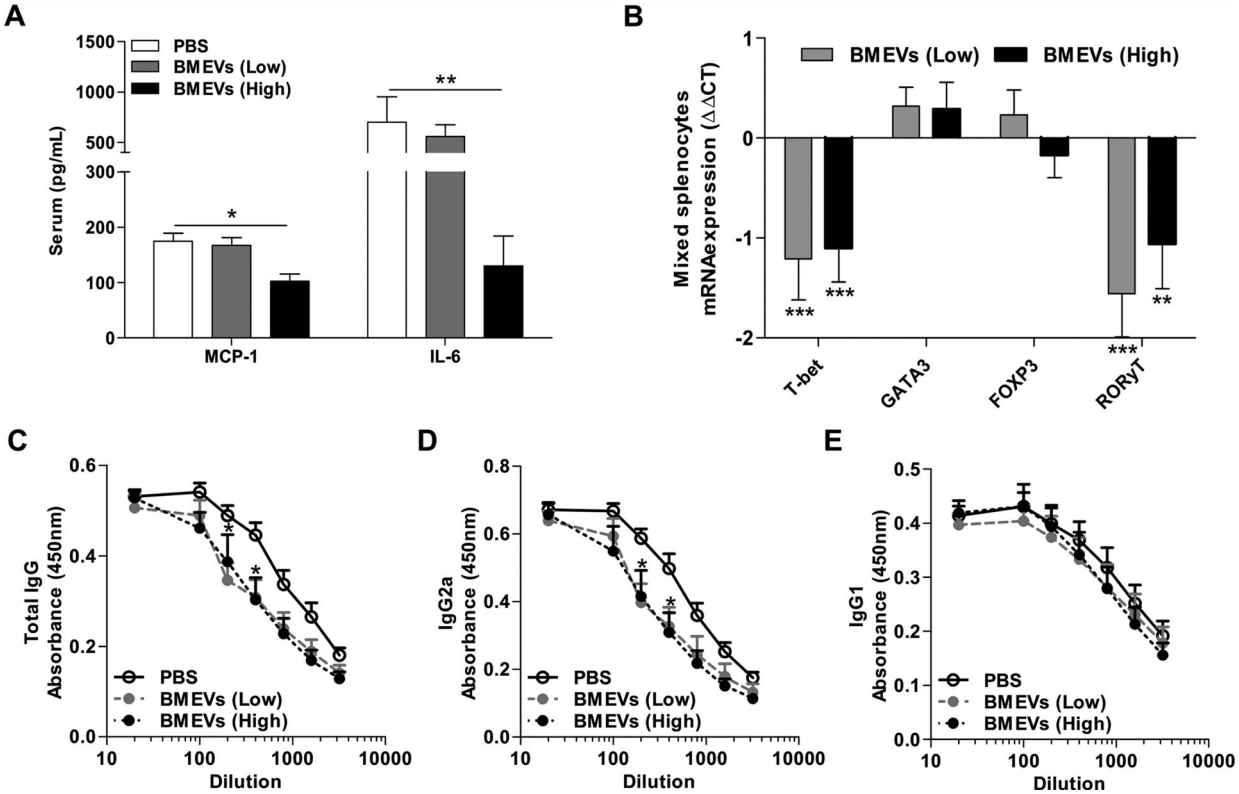

Figure 6. Functional change in CIA model by BMEVs. Serum levels of MCP-1 and IL-6 were determined by multiplex bead array assay. (A). Expression of T-bet, GATA-3, Foxp3, and RORγT mRNA in isolated splenocytes measured by RT-qPCR (B). Serum levels of total IgG, IgG2a, and IgG1 quantified by ELISA (respectively, C, D, and E). Data are represented as mean ± SEM ($n = 10$) and statistical differences were calculated using Mann–Whitney *U* test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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The authors have declared no conflict of interest.

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Chapter 5

Milk-derived extracellular vesicles as a RNAi-based drug for arthritis

Submitted for publication

Chapter 5

Milk-derived extracellular vesicles as a RNAi-based drug for arthritis

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Submitted for publication

Abstract

Rheumatoid arthritis (RA) is a systemic disease, considered the most common form of chronic autoimmune inflammation. The true origin of RA is still unknown; however, different causes are associated, such as diet composition. The use of diet components as alternative therapies may reduce the intake of small chemical drugs and limit unwanted side effects provided by medicines. Studies have already shown the potential benefits of extracellular vesicles (EVs) on the immune response. These particles are composed by mRNAs and miRNAs, stimulating target cells at pos-transcriptional level and emerging as a tool to use in disease gene therapy. It was detected EVs in different body fluids, including the milk independent of origin. The bovine milk is still highly consumed by human societies all over the world, even after the breastfeeding period. New data based on milk EVs content suggest that this type of food could be a regulator in inflammatory diseases based on miRNA presence. EVs are highly stable and resistant to RNAse activity, extreme pH and temperature, akin to what was observed in milk, indicating that these molecules could be transferred to other cells and be functional in its new location. Recently our group demonstrated some immunoregulatory proprieties of bovine-derived milk EVs on immune cellls and in two arthritis murine models. These EVs may regulate T cell differentiation and cytokines production, contributing to the attenuation of arthritis response. Moreover, some miRNA present in the milk EVs are related to an anti-inflammatory response, and possibly could regulate the inflammatory response of arthritis after intake and consequently be used as a therapy for arthritic patients. Therefore, we believe and propose that bovine milk EVs are safe natural components that could be a potential adjuvant therapy for patients with arthritis.

Introduction

Rheumatoid arthritis (RA) is a systemic disease, considered the most common form of chronic autoimmune inflammation. This disorder affects primarily the synovial joints, leading to destruction of cartilage and bone, causing pain, swelling and stiffness and, consequently, loss of function [1]. The causes of RA may include infection, genetic, endocrine, immunological or psychogenic changes, however, its true origin still remains unknown [1]. Studies have suggested that environment factors are important contributors for the development of arthritis. Factors as smoking, obesity and physically demand job were related to increased risk of RA [2]. In addition, regular consumption of sugar-sweetened soda, but not diet soda, was also associated with increased RA risk in women [3]. On the other hand, alcohol consumption, specifically wine, as well as physical activity, presented an inverse association with RA risk [2]. Although there are still controversial data about omega-3 intake and RA, some studies showed a positive correlation with fish consumption and the reduction of RA risk in woman at long-term [4] or suppression of clinical symptoms in RA [5]. Therefore, these studies indicate that the food composition could influence the arthritis risk and, consequently, its inflammatory response.

Despite distinct inductors of RA, there is a common denominator, the inflammation. The inflammatory process showed in RA is characterized by the production of inflammatory mediators and recruitment of leukocytes to the synovial joint [6]. The synovium contains myeloid and dendritic cells that secrete cytokines necessary for the activation of T lymphocytes. T cells can represent up to 50% of the cells of the synovial membrane of a patient with RA and, once activated, produce pro-inflammatory cytokines and induce angiogenesis, synovial hyperplasia and differentiation of osteoclasts [6]. Although RA is considered lymphocyte mediated, other cells may also contribute releasing inflammatory mediators, such as monocytes, macrophages and synovial fibroblasts. Since these cells interact with each other, biotech drugs have been developed, which are innovative therapeutic agents with specific action, including the blockage of specific cytokines or their receptors involved in the immune process [7, 8].

Although, the use of anti-inflammatory drugs has a positive effect on arthritis [7, 8], their use at long-term could result in adverse effects. Thus, the use of alternative

therapies may reduce the intake of small chemical drugs and limit unwanted side effects. In the last decades, the investigation about the influence of extracellular vesicles (EVs) on immune cells and inflammatory mediators has emerged these particles as modulators of immunity and, therefore as putative therapeutic tool [9]. EVs are membrane vesicles of endosomal or plasma membrane origin released from cells into the extracellular environment, called exosomes and microvesicles, respectively [10]. Several studies have suggested that EVs, which contain proteins, mRNA and microRNA (miRNA), stimulate target cells via surface receptor activation [11] or genetic transfer into cells [12]. MiRNAs are small RNA molecules containing about 20 nucleotides, which regulate the levels of protein expression by promoting mRNA degradation or attenuate protein translation in the post-transcription phase [13]. These molecules are described to have potential applications in the disease gene therapy.

The hypothesis

We hypothesize that with the intake of milk EVs, bovine miRNAs regulate the inflammatory process that is characteristic of arthritis, through targeting mRNAs that are involved in the immune system development and function. Recent studies have shown the presence of EVs in food derived from mammals and plants [14, 15]. Since the EVs are known to be mediators of immunity, we suggest that these food components may act as regulatory agents that may treat or prevent several inflammatory diseases. Milk is considered the first food consumed by mammals, including humans, and the description of it as a food rich in EVs initiated the research to study the effect of its EVs over health and disease [15]. Our group has recently demonstrated that bovine milk-derived EVs have immunoregulatory properties on immune cells in vitro and in two arthritis murine models [16, 17]. In this context, the intake of small particles, extracellular vesicles provided from food, could be a new therapeutic form and natural than the use of medicines to treat RA patients.

Evaluation of the hypothesis

The milk provided by mothers is a source of nutrition for infants, mainly newborns, which contains a mixture of macronutrients and micronutrients. Furthermore, the milk has immune competent cells and soluble proteins [18], which provide protection

against infections. Other function of milk is to induce immune tolerance [19] and provide protection against developing allergies [20]. The milk has also other bioactive molecules, such as miRNAs. Recently, miRNAs have been studied in order to find out the cause of various diseases and to search for new therapies. As example, the miR-146a, which is upregulated in rheumatoid arthritis in peripheral mononuclear cells [21] and synovial tissue [22]. In addition, the deletion of specific miRNA, such as miR-155 in mice, eliminates the disease in collagen-induced arthritis model [23]. As in other biological fluids, microvesicle-like particles are present in mouse [24] and human milk [25]. mRNAs and miRNAs have also been isolated from bovine milk-derived microvesicles [15, 26]. Detection of milk protein mRNAs in cultured cells indicated the transfer of microvesicles contents, such as miRNA, into cells, supporting the notion that the milk EVs intake may contribute to the presence and availability of functional miRNA in the body [26].

It is well described that RNA content of the milk microvesicles could be transferred to other cells and be functional in its new location. These microvesicles may, therefore, generate gene transfer, such as from mother to newborn during breastfeeding [27]. Although, the milk given to the newborn is essential for development of the immune system, there are still many doubts in relation to the role of milk consumption in the immunity, mainly provided from cows, post breastfeeding. Study from Sun et al. [15] showed that vesicles stemmed from colostrum of cow's milk contain higher concentrations of immune-related miRNAs and may contribute to immune modulating characteristics when administered to cells in culture. The miRNA may be detected in plasma, serum, urine, saliva, milk and other body fluids in mammals. Moreover, analysis of circulating RNA indicated that these molecules are highly stable and resistant to RNAse activity, extreme pH and temperature, akin to what was observed in milk [28], indicating the resistance to the similar conditions present in the gastrointestinal tract. Recently, it was demonstrated that the miRNAs present in the milk was also detected post-prandial in the serum of humans and mice after milk consumption [29]. Two miRNAs were described, the miR-29b and miR-200c. These data indicate that miRNAs from other species, instead of be excreted, may be absorbed by human gastrointestinal tract and act in the regulation of genes in other sites through the same targets. It can be possible only because miRNAs show a large phylogenetic overlap and are evolutionary conserved [30].

Cross-sectional studies suggest that the consumption of dairy products is inversely correlated with low-grade systemic inflammation [31, 32], indicating a beneficial effect provided from different but indistinct sources. However, recent study from Michaëlsson et al. [33] analyzed data of over a period of 20 years, associating milk intake with higher mortality and hip fractures. Despite this study showed the positive relation between milk intake and increase in IL-6 and 8-iso-PGF2α, it is highlighted that the consumption of fermented milk (soured milk and yogurt) has an inverse relation, being anti-inflammatory. Moreover, they indicate that the negative action of milk intake would be due to D-galactose presence, since this component is reduced in cheese and fermented products. Other described components to increase the inflammation and allergenicity are milk proteins, mainly casein [34]. However, this component is also reduced in soured milk and partially digested in yogurt, avoiding the intolerance and consequently inflammation. Indeed, some rheumatic symptoms in patients with RA are allergic food related, showing an increase in Ig immune complexes after the consumption of food [35]. Milk allergic patients when challenge with it show exacerbation of inflammatory arthritis with immunologic hypersensitivity [36]. An allergic reaction often dampens autoimmunity through Th2 activation. Therefore, the recognition and immune response to milk proteins appears be dependent miRNA regulation, once it is observed the participation of the same miRNA in different T helper cell differentiation [37]. Other milk components, that could be still present in dairy products, may have an anti-inflammatory effect and maybe the use of them could prevent any antigen reaction related to allergy and also RA. As milk EVs are stable under different environment conditions, we suggest that the anti-inflammatory response is, at least in part, caused by these vesicles.

The benefits of milk consumption to reduced osteoarthritis progression and alleviation of the symptoms has previously been shown. In fact, studies with symptomatic osteoarthritis patients demonstrated that the effect at short- (6 weeks) and long-term (48 months) of milk intake is amelioration in the discomfort caused by the disease [38-40]. These evidences related to osteoarthritis indicate that maybe components of milk could modulate also the inflammation as observed in RA or at least, alleviate its symptoms. It is known that miRNAs are present and stable in synovial fluid of patients with RA, and show a distinct profile from plasma miRNAs [41]. MiRNAs described to be upregulated in the joint fluid, synovial and periferal

blood nuclear cells of RA are miR-146a, miR155, miR-223 and miR16 [21, 22, 41]. These miRNAs are not in huge amount in the mature milk or colostrum, and the miR-21, which is considerably present in milk [15], is downregulated in RA [42]. In addition, the miR-21 was also related with regulation of TLR4 via PDCD4, and downregulation of NF-κB, indicating its anti-inflammatory potential [43]. It was already suggested that the consumption of milk may be involved in the increased expression of miRNA-155, also present in the EVs, responsible for T regulatory cells maturation [44]. It shows the possible contribution of milk to an anti-inflammatory response. Thus, the inflammatory response of arthritis after intake of milk EVs possibly could be regulated and consequently be used as a therapy for these patients.

Our group has recently demonstrated that bovine milk-derived EVs (BMEVs) have immunoregulatory properties on immune cells *in vitro* and in two arthritis murine models [16, 17]. These particles were isolated from commercial milk and characterized by the presence of miRNAs and bioactive TGF-β [16]. The cytokine TGF-β is known to be involved in the regulation of T cells differentiation with suppression of Th1 and Th2 cells, and depending of the presence of other cytokines, promotes Th9, Th17 and Foxp3+ regulatory T cells [45]. We also observed that the *in vivo* treatment with BMEVs in a model of collagen-induced arthritis (CIA) and in IL-1Ra-deficient mice attenuated the arthritis response, indicating a possible anti-inflammatory effect of these vesicles [17]. According to the data, it occurs a splenic reduction of Th1 and Th17, and also pro-inflammatory cytokines (IL-6 and MCP-1) in the serum. Moreover, we suggest that BMEVs could be able to stimulate Treg differentiation [17]. Therefore, BMEVs, which is still present in commercial milk, appear to be an interesting tool to be tested in RA patients, since it is already consumed in milk form by a large population, but it has not yet been described the specific effect of BMEVs in arthritic patients.

Supporting the use of food EVs in the gene therapy, recent study from Mu et al. [14] showed the effect of plant derived exosome-like nanoparticles (EPDENs) on mammalian cells. These particles were isolated from four edible plants (grapes, grapefruit, ginger and carrot) and their content consists of proteins, lipids and miRNAs. In the intestine, the EPDENs are taken up by macrophages and stem cells, inducing expression of genes for anti-inflammatory cytokines, antioxidation, and

activation of Wnt signaling, which are crucial for maintaining intestinal homeostasis. These data suggest that there is an interspecies communication between EPDENs and mammalian cells. Moreover, Lukasik and Zielenkiewicz [46] showed that some miRNAs molecules derived from plants are abundant in human and porcine breast milk, indicating that the milk composition may be related to food intake. Controversially, it was also suggested by other authors that the cellular uptake of EVs derived from plants and mammals could be different interspecies, affecting negatively the uptake of plant exosomes by human intestinal cells [29]. Despite these opposite ideas, it is getting clearer in the literature that the genetic information from food and what we choose to eat may be important to influence the improvement of diseases response.

Since miRNAs represent a novel class of signaling molecules for intracellular regulation, the investigation about the effects of EVs derived from food is increasing. The bovine milk is still highly consumed by human societies all over the world, even after the breastfeeding period. The gastrointestinal tract may represent a functional pathway for absorption of food-derived miRNAs, since it was described that some milk miRNAs are found to be increased in the blood circulation. The EVs can be, in the future, considered as a nutrient that provides the delivery of therapeutic miRNAs into the body and consequently contributing to the regulation of immunity. After determination of beneficial effects of milk EVs in arthritis models, it would be interesting to verify our hypothesis whether the consumption of milk EVs also regulates arthritis in clinical patients.

Conclusion

Studies have already shown the potential benefits of EVs on the immune response. New data based on milk EVs content suggest that this type of food could be a regulator in inflammatory diseases based on miRNA presence and bioactive molecules. We believe that milk EVs are safe natural components that could be a potential adjuvant therapy for patients with arthritis.

Conflict of interest statement

None.

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Chapter 6

Milk extracellular vesicles accelerate osteoblastogenesis but impair bone matrix formation

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Milk extracellular vesicles accelerate osteoblastogenesis but impair bone matrix formation☆

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Abstract

The claimed beneficial effect of milk on bone is still a matter for debate. Recently extracellular vesicles (EVs) that contain proteins and RNA were discovered in milk, but their effect on bone formation has not yet been determined. We demonstrated previously that bovine milk-derived EVs (BMEVs) have immunoregulatory properties. Our aim was to evaluate the effect of BMEVs on osteogenesis by mice and human mesenchymal stem cells (hMSCs). Oral delivery of two concentrations of BMEVs to female DBA/1J mice during 7 weeks did not alter the tibia trabecular bone area; however, the osteocytes number increased. In addition, the highest dose of BMEVs markedly increased the woven bone tissue, which is more brittle. The exposure of hMSCs to BMEVs during 21 days resulted in less mineralization but higher cell proliferation. Interestingly BMEVs reduced the collagen production, but enhanced the expression of genes characteristic for immature osteoblasts. A kinetic study showed that BMEVs up-regulated many osteogenic genes within the first 4 days. However, the production of type I collagen and expression of its genes (*COL1A1* and *COL1A2*) were markedly reduced at days 21 and 28. At day 28, BMEVs again lead to higher proliferation, but mineralization was significantly increased. This was associated with increased expression of sclerostin, a marker for osteocytes, and reduced osteonectin, which is associated to bone matrix formation. Our study adds BMEVs to the list of milk components that can affect bone formation and may shed new light on the contradictory claims of milk on bone formation.

Keywords: Extracellular vesicles; Milk; Bone; Osteogenic differentiation; Bone matrix

1. Introduction

Bone is a dynamic tissue that continuously renews itself in a coordinated process of bone formation by osteoblasts and bone degradation by osteoclasts [1]. Osteoblasts cells are derived from mesenchymal stem cells (MSCs), and besides bone formation, they also regulate bone resorption by osteoclasts [2]. Diverse factors could influence bone formation, including nutrition by providing vitamin-D and essential calcium for mineralization [3,4]. Milk has nutritional value, is rich in calcium and contains other components, such as lactoferrin and whey protein, that may enhance osteoblast differentiation [5,6]. In respect to bone health, the consumption of milk is mainly associated with improved bone density [7–9]. Other studies controversially demonstrated a positive correlation between milk consumption and higher fracture risk [10], reduction of bone remodeling [11] and not even reducing fractures in osteoporosis patients [12].

In recent decades, the extracellular vesicles (EVs) aroused the interest of researchers on their use as modulators of immunity [13]. The description of milk as a source of EVs initiated the study of their implications [14]. Indeed, our group has recently demonstrated that bovine milk-derived EVs (BMEVs) have immunoregulatory properties on immune cells *in vitro* and in two arthritis murine models [15,16]. EVs are membranous vesicles released by cells into the extracellular environment [17]. They contain proteins, mRNA and microRNA (miRNA) that stimulate target cells through the activation of surface receptors [18] or gene transfer to other cells [19]. Moreover, encapsulated in EVs, these molecules are protected against protease and RNAse activity. We found that BMEVs are stable at extreme pH [15] and thereby potentially can resist the harsh conditions in the gastrointestinal tract.

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In this study, we aimed to investigate the effect of BMEVs on osteoblast differentiation using female DBA/1J mice and human bone marrow-derived mesenchymal stem cells. We demonstrated that BMEVs increased osteoblast differentiation by accelerating a transition into osteocytes. However, these effects are associated with a substantial impairment of bone matrix formation *in vitro* that may explain the higher presence of woven bone in mice.

2. Materials and methods

2.1. Isolation of extracellular vesicles from commercial milk

BMEVs were isolated from commercial semi skimmed cow milk as previously described [15]. Shortly, milk was centrifuged at 70.000 g for 1 h at 4 °C, and part of the supernatant was filtered. Thereafter, the effluent was filtered with a syringe filter (0.2 μm). This effluent was centrifuged at 110.000 g for 1.5 h at 4 °C. The pellet was dissolved in PBS. The amount of protein was measured with a Micro-BCA kit (Thermo Scientific, Pierce, Rockford, USA). Its characterization was done previously by dynamic light scattering (DLS), determination of proteomic and miRNA content [15,16], accordingly to the International Society of Extracellular Vesicles [20].

2.2. Mice treatment

Female 10–12-week-old DBA/1J mice (Janvier, Elavage, France) received in the drinking water BMEVs in two different particles concentrations: 4.7×10^6/mL (low) or 14.3×10^6/mL (high) during 7 weeks. All mice were housed in filter-top cages, and a standard diet and water were provided *ad libitum*. The body weight was evaluated once a week and the drinking water consumption three times a week. The *in vivo* studies complied with national legislation and were approved by the local authorities on the care and use of animals (DEC-nr:2014–083).

2.3. Histological analysis

Murine proximal tibias were isolated, fixed in 4% phosphate buffered formalin (pH 7.4) and decalcified in 5% formic acid. Tibias were dehydrated with an automated tissue processing apparatus (LeicaASP300, Rijswijk, The Netherlands) and embedded in paraffin, and sections of 7-μm thickness were obtained. For analysis of trabecular bone area, density of osteocytes per trabecular bone area and adipocytes area in the bone marrow, the sections were stained with hematoxylin and eosin. The percentage of trabecular bone in sections of proximal epiphysis and metaphysis of the tibia was determined by histomorphometry. This variable was determined at ×20 magnification using a micrometer containing a 121-point grid. Fields were chosen at 1 mm from the epiphyseal plate and articular cartilage. The grid was superimposed above (epiphysis) and under (metaphysis) the growth plate for a total of 4 and 10 fields per section, respectively [21]. The percentage of total adipocytes area in the bone marrow was determined in the tibia with ImageJ software (NIH Image).

For determination of lamellar and woven bone area, Alcian Blue-Hematoxylin/Acid Fuchsin-Eosin staining was used as previously described [22]. Cartilage is stained blue, woven bone mauve-blue and mature bone mauve. The percentage of lamellar bone and woven bone was determined in the total trabecular bone area in the proximal epiphysis and metaphysis of the tibia with Adobe Photoshop C6 software (Adobe Systems, Inc.) and ImageJ software (NIH Image).

2.4. Human mesenchymal stem cells

Human fetal bone marrow-derived mesenchymal stem cells (hMSCs) were purchased from ScienCell Research Laboratories (Cat. #7500; Carlsbad, CA, USA). Cells were cultured in MSCGM™ BulletKit* (PT-3001; MSCBM™, MSCGM™ Single-Quots* Kit, Lonza Walkersville, Inc., Walkersville, MD) at 37 °C in a humidified atmosphere of 5% CO_2. hMSCs were used between Passages 3 through 5.

2.5. Osteogenic differentiation and treatment

Human MSCs were seeded at an initial density of 10,000 cells/cm^2 in a 24-well plate with DMEM-F12 containing 10% exosome-free FBS and penicillin/streptomycin and were incubated at 37 °C in a humidified atmosphere. FBS extracellular vesicles-free was obtained after centrifugation at 100.000 g for 14 h at 4 °C with supernatant collection. After 2 days, the culture medium was supplemented with 0.1-μM dexamethasone, 45-μM ascorbic acid and 10-mM β-Glycerophosphate purchase from Sigma-Aldrich (St Louis, MO, USA). Cells were treated with the osteogenic-differentiating medium in the control group and experimental groups; in the latter group, BMEVs were added at the time points indicated in the Results section. The culture medium was replaced every 3–4 days.

2.6. Cellular uptake of BMEVs

BMEVs were stained with PKH67 Green Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Sigma-Aldrich, St. Louis, MO, USA) as described previously [15]. Briefly, PKH67-labeled BMEVs were taken up in culture medium. PKH67-labeled BMEVs (20 μg/mL) were incubated with hMSCs for 24 h at 37 °C, or 4 °C as control, with 5% CO_2. After incubation, cells were washed twice, fixed with 4% formaldehyde and stained with 4,'6′-diaminido-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). Cellular uptake of BMEVs was observed using a fluorescent microscope (Leica microscopy, Rijswijk, The Netherlands).

2.7. Histochemical staining

Mineralization was evaluated of osteogenic differentiation using Alizarin red staining. Cells were washed with PBS and fixed with 4% formaldehyde for 30 min and stained for 45 min with a 2% solution of Alizarin red (w/v in distilled water) pH = 4.3. Thereafter, cells were washed with water and visualized under a light microscope. Stained calcium deposits were extracted with 500 μl of 0.1 N sodium hydroxide, recording the optical density of 540 nm.

Determination of collagen production by the cells was performed using a solution containing 0.2 ml saturated picric acid in distilled water, 0.1% Fast green FCF (noncollagenous proteins) and 0.1% Sirius red (collagen) as described before [23]. The plates were incubated for 30 min at room temperature in a shaker protected from the light. Wells were rinsed several times with distillated water and visualized under a light microscope. The color was extracted with 500 μl of 0.1 N sodium hydroxide in absolute methanol and measured at wavelengths 540 nm correspondent to Sirius red staining.

2.8. Cell proliferation assay

The amount of cells was determined by a crystal violet mitogenic bioassay. Cells were washed with PBS and stained with 0.1% crystal violet/25% methanol for 20 min. After washing with water, the cells were observed under the microscope. Crystal violet in the cells was extracted using 10% acetic acid in distillated water, and absorbance was read at 540 nm.

Cell number was also assessed at day 21 using colorimetric XTT assay (Roche Diagnostics, Indianapolis, IN, USA) according to manufacturer's instructions. The colorimetric change was measured at 0, 4, 8 and 24 h after the addition of substrate, and absorbance was read at 450 nm.

2.9. Alkaline phosphatase enzymatic activity

After 21 days of osteoblast differentiation, cells were washed twice with ice-cold PBS and incubated for 30 min at 4 °C with assay buffer (pH 9.0, 1.5-M Tris–HCl, 1-mM $ZnCl_2$, 1-mM $MgCl_2$ and 1% Triton X-100) and collected by scraping. Cell lysates were centrifuged at 1000 g for 10 min at 4 °C. One hundred fifty microliter of the substrate solution [pH 10.0, 7.6-mM 4-nitrophynyl phosphate disodium salt hexahydrate (Sigma-Aldrich), 100-mM Tris–HCl and 10-mM $MgCl_2$] was added to 50-μl supernatant and incubated for 1 h at 37 °C. The reaction was stopped by adding 50-μl 3-M NaOH, and the absorbance was read at 410 nm. ALP activity was normalized for total protein levels measured by Micro-BCA kit (Thermo Scientific, Pierce, Rockford, USA).

2.10. Quantitative real-time polymerase chain reaction (qRT-PCR)

mRNA was isolated from cells using the TRIzol method (Invitrogen, Carlsbad, CA, USA). Isolated RNA was treated with DNaseI (Invitrogen, Carlsbad, CA, USA) to remove DNA contamination. Synthesis of cDNA was accomplished by reverse transcription PCR an oligo (dT) primer and Moloney murine leukemia virus Reverse Transcriptase (Applied Biosystems Inc., Foster City, CA, USA). Quantitative real-time PCR was performed using SYBER-Green on a Step-One according to the manufacturer's instructions (Applied Biosystems Inc., Foster City, CA, USA). The cycle of threshold (C_t) for each sample was normalized to GAPDH expression. The gene expression was determined by the $2^{-\Delta\Delta Ct}$ method. The primer pair sequences are listed in Supplementary Table 1.

2.11. Statistical analysis

Data are expressed as the mean ± S.E.M. All data were analyzed for normality of distribution using Kolmogorov–Smirnov test and were found to be normal. Multiple comparisons were performed using one-way ANOVA followed by Newmann–Keuls test. Student's *t* test was used for comparisons between two groups. The statistical analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Statistical significance was stated as follows: $^{*}P<.05$; $^{**}P<.01$; $^{***}P<.001$.

3. Results

3.1. Oral delivery effect of BMEVs on the tibia bone of mice

Oral delivery of BMEVs in two different concentrations during 7 weeks did not change the trabecular bone area (Fig. 1A); however, it increased in both BMEVs groups the osteocytes number (Fig. 1B and E). In order to analyze the stage of bone formed, we performed the Alcian Blue-Hematoxylin/Acid Fuchsin-Eosin staining. The highest concentration of BMEVs increased the percentage of woven bone compared with PBS group (Fig. 1C and E). In addition, a reduction of adipocytes area in the bone marrow of mice that received the high concentration of BMEVs was also observed (Fig. 1D and E). No differences were observed between the groups in relation to final body weight (PBS: 21.72 g ± 1.10; BMEVs low: 21.86 g ± 0.44; BMEVs high: 21.64 g ± 0.45; $P>.05$) and drinking water consumption (PBS: 4.76 ± 0.29; BMEVs low: 4.88 ± 0.34; BMEVs high: 4.70 ± 0.19 ml/day/mice; $P>.05$).

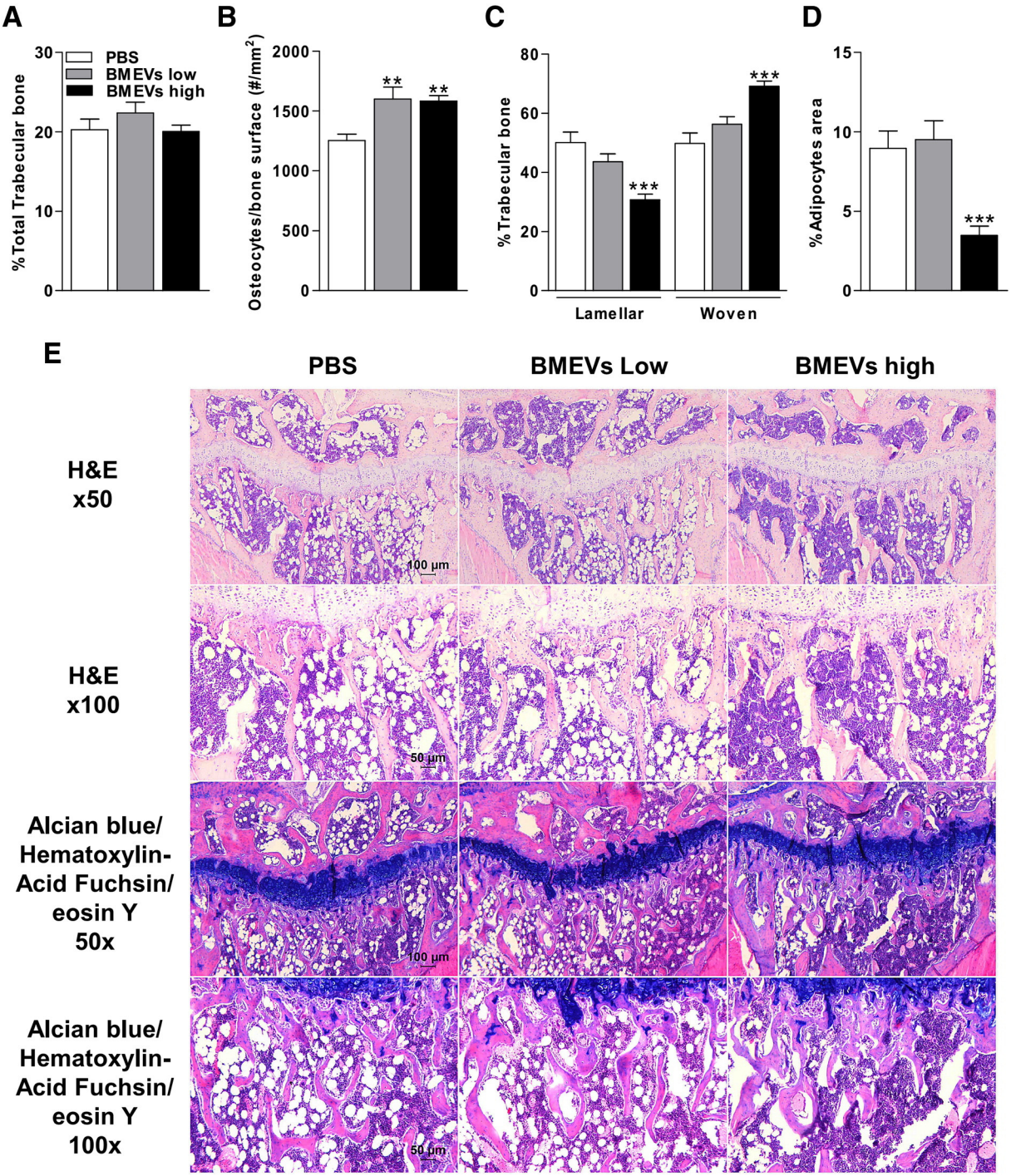


Fig. 1. Mice treated with BMEVs in two different concentrations (4.7×10^6/mL (low) or 14.3×10^6/mL (high)) by oral delivery in the drinking water during 7 weeks. (A) The percentage of trabecular bone and (B) number of osteocytes in the proximal epiphysis and metaphysis of the tibia by hematoxylin–eosin staining. Quantification of (C) lamellar bone (mauve) and woven bone (mauve-blue) by Alcian Blue-Hematoxylin/Acid Fuchsin-Eosin staining. (D) Percentage of adipocytes area in the bone marrow. (E) Representative histology of proximal tibia in each group. Bars represent the mean ± S.E.M. ($n=5$–7). $^{**}P<.01$, $^{***}P<.001$ *vs.* PBS group.

3.2. Uptake and dose finding of BMEVs on osteoblast differentiation in hMSCs culture

Next, we evaluated whether the osteoblastogenesis of hMSCs may explain the higher presence of osteocytes and woven bone in mice treated with BMEVs. First, the uptake of BMEVs by hMSC was assessed using a fluorescent dye labeled with the vesicles. Uptake of BMEVs was detected by hMSCs 24 h after incubation (Fig. 2A). Then we performed a BMEVs dose–response experiment. Human MSCs were cultured in osteogenic medium supplemented with 2-, 20- or 200-μg/ml BMEVs during 21 days. Human MSCs treated with 200 μg/ml of BMEVs showed less mineralization as observed by Alizarin red staining (Fig. 2B and C) albeit a higher amount of cells by crystal violet staining (Fig. 2D) compared to the control without BMEVs. Therefore, it was decided to use a concentration of 200-μg/ml BMEVs (about 10% of the EVs concentration measured in commercial milk) in the next analysis.

3.3. Effect of BMEVs treatment during 21 days on hMSCs differentiating into osteoblasts

A higher number of cells in hMSCs treated with BMEVs as analyzed by XTT (Fig. 2E) supports the data obtained with crystal violet staining at day 21 (Fig. 2D). Besides reduced mineralization, also the amount of collagen deposition was reduced in the BMEVs-treated cells compared to control, as observed with Picrosirius red staining (Fig. 2F). Accordingly, the ratio of collagen/cells (Fig. 2G) and the expression of collagen 1α1 and collagen 1α2 genes (Fig. 2H) were also reduced in the BMEVs group as compared to the control. Although, BMEVs treatment showed lower mineralization, the ALP enzymatic activity was higher than in the control group (Fig. 2I).

The expression of osteogenic genes in the hMSCs was also altered after 21 days of BMEVs exposure (Fig. 2J). The expression of early osteoblastic differentiation genes, Runt-related transcription factor 2 (*RUNX2*) and alkaline phosphatase (*ALP*), was increased, while the elevated osterix (*OSX*) expression did not reach statistical significance in the cells treated with BMEVs. The *ALP* expression was in agreement with the higher enzymatic activity of ALP (Fig. 2I). Osteocalcin, which is involved in the extracellular matrix formation, was inhibited by BMEVs. Osteopontin, a gene related to regulation of bone mineralization, was higher in BMEVs group compared to control. The *RANKL* and osteoprotegerin are genes that indicate the osteoblast capacity to regulate osteoclast bone resorption. The *RANKL* expression was increased after BMEVs treatment, without alterations in osteoprotegerin. In order to unravel which pathways could be responsible for the increased expression of *RUNX2*, the expression of transforming growth factor beta 1 and 2 (*TGF-β1* and *2*), bone morphogenetic protein-2 (*BMP-2*), fibroblast growth factor-2 (*FGF-2*), WNT1-inducible-signaling pathway protein 1 (*WISP-1*) and axis inhibition protein 2 (*AXIN2*) was evaluated. Only *FGF-2*, a growth factor related to cell proliferation and *WISP-1*, a product of the Wnt signaling pathway, were increased after 21 days of BMEVs treatment.

3.4. Kinetic of BMEVs on osteoblast differentiation

In a different set of experiment, we verified the effect of BMEVs treatment on osteoblast differentiation over time. Fig. 3A and B shows the mineralization and quantification by Alizarin red staining extraction, respectively. Despite BMEVs' induced lower mineralization after 21 days, the calcium deposition was increased at day 28 compared to control. However, the amount of mineralized nodules number was reduced in BMEVs group treated during 28 days (Fig. 3C). On both day 21 and 28, an increase in cell number by BMEVs was found (Fig. 3D). Moreover, cells treated with BMEVs for 21 and 28 days showed lower collagen deposition by Picrosirius red staining as compared to the control group (Fig. 3E).

At day 21, the MSCs exposed to BMEVs expressed predominantly markers characteristic for immature osteoblasts; however, at day 28, these cells expressed sclerostin (*SOST*), a specific protein produced by osteocytes (Fig. 3F). In addition to the *SOST* gene, the expression of other osteogenic genes during hMSCs differentiation was altered over time by the presence of BMEVs (Fig. 3G-M). At early time point (4 and 7 days), an increase in *RUNX2*, *COL1A1*, osteopontin, *FGF-2* and *WISP-1* expression induced by BMEVs was observed. This effect on early gene expression was not seen when hMSCs were exposed to the same numbers of 100-nm Latex beads, nor when were BMEVs preheated that denatured proteins and degraded miR-29 (Supplementary Fig. 1).

At day 14, only *WISP-1* expression was increased compared to control. *RUNX2* and *FGF-2* followed the same pattern, showing an increase in days 21 and 28 after BMEVs treatment. At day 21, osteopontin expression was markedly increased with no alterations in osteocalcin. However, the opposite was observed at day 28, an increase in osteocalcin and no alteration in osteopontin expression. Interestingly, *COL1A1* and *COL1A2* expression was reduced with 21 and 28 days of BMEVs treatment (Fig. 3H and I), and this is fully in line with the observed reduced amount of collagen protein (Fig. 3E). Furthermore, the expression of osteonectin, a matrix protein described to link mineral to collagen [24], was also reduced at day 28 (control: 4.41 ± 0.25 *vs.* BMEVs: 0.57 ± 0.16 mean ± S.E.M.; $P < .001$).

3.5. The effect of timing and duration of BMEVs challenge of hMSCs on osteoblast differentiation

Fig. 4 shows the effect of BMEVs exposure of MSCs started at day 0 for different durations on osteoblastogenesis at day 28. Interestingly, according to the quantification of mineralization, the presence of BMEVs from day 0 until day 14 was enough to increase mineralization (Fig. 4A and B). Only maintaining BMEVs for a minimum of 21 days were able to increase cell amount (Fig. 4C). The ratio of collagen/cells (Fig. 4D) demonstrated a reduction of collagen deposition at the same time points related to increased mineralization (from day 14 to 28). Accordingly to these data, *RUNX2*, osteocalcin and *FGF-2* expression were increased, and *COL1A1* was reduced at the same time points and only needed until day 14 of milk EV exposure (Fig. 4E). *WISP-1* showed a biphasic pattern unrelated to what was observed by Alizarin red staining, and a similar trend was seen with osteopontin (Fig. 4E).

In an inverted experimental design, hMSCs were challenged with BMEVs starting at different time points of culture until the end at day 28. Mineralization (Fig. 5A–B) was increased by BMEVs when added for 21 days or longer. The same effect was seen for cell number, although starting at day 14 still had a moderate effect (Fig. 5C). The ratio of collagen/cells was reduced accordingly the time that BMEVs were added in the medium compared to the control group (Fig. 5D). The gene expression of *RUNX2*, osteocalcin, *COL1A1* and *FGF-2* showed differences only in hMSCs challenged with BMEVs started from the beginning as compared to control. *WISP-1* was increased when BMEVs were added at day 4 and day 0. Osteopontin expression did not change in any condition (Fig. 5E).

3.6. Characterization of BMEVs on osteoblast differentiation

Proteomic analysis of BMEVs [16] revealed the presence of classical components of milk, such as lactoferrin, lactoalbumin, lactoglobulin and casein proteins that are positively associated to osteoblast differentiation (*in vitro*) and bone formation (*in vivo*), whereas, for example, apolipoprotein E and osteopontin are negatively associated (Supplementary Table 2). Members of the miR-29 family (miR-29a, −29b and −29c) posttranscriptionally regulate gene expression in osteoblasts, thereby controlling differentiation [25,26]. PCR analysis

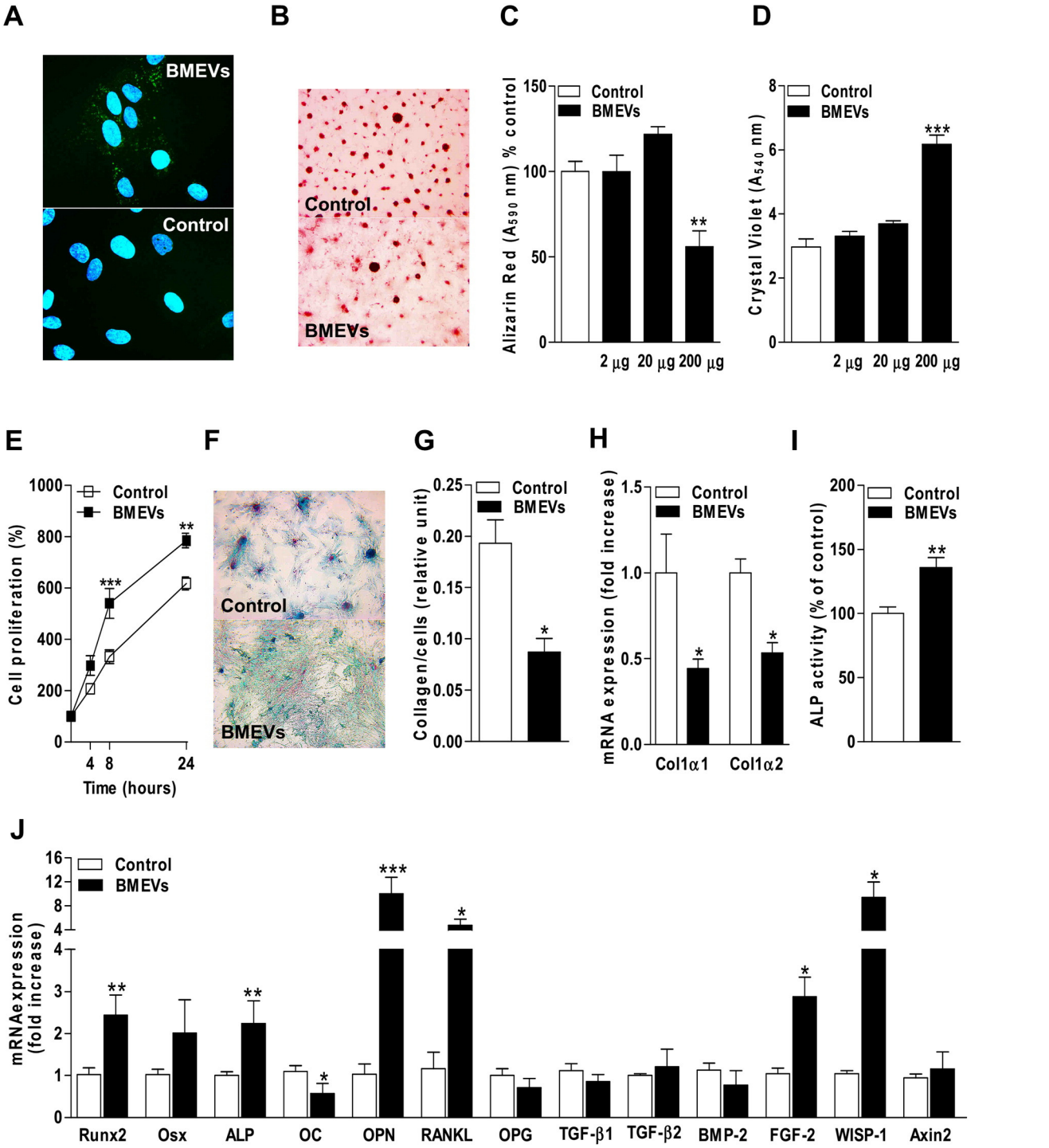
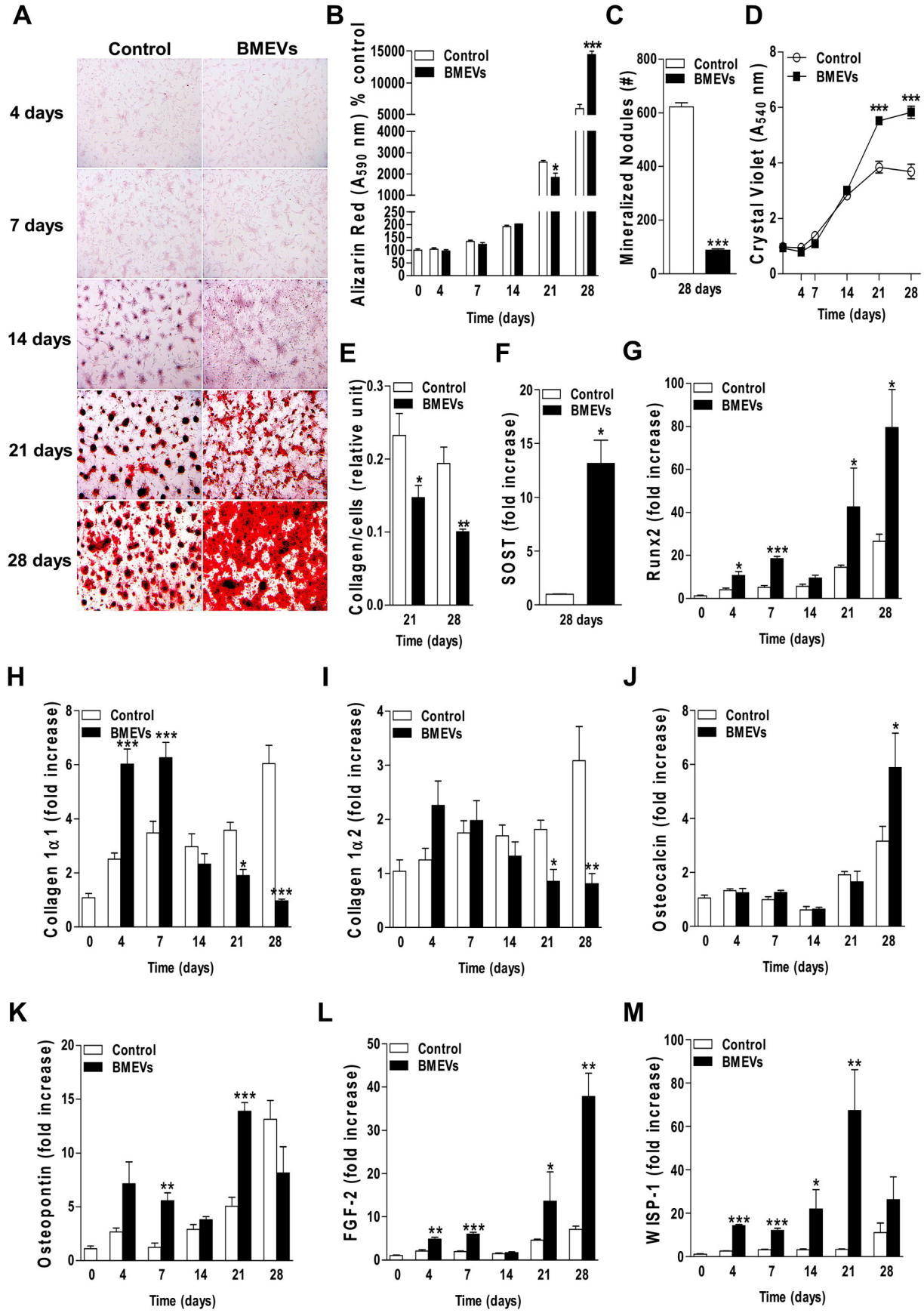


Fig. 2. Uptake of bovine derived-milk extracellular vesicles (BMEVs) by human mesenchymal stem cells (hMSC), dose finding of BMEVs and effect of BMEVs treatment on hMSC differentiated into osteoblast for 21 days. (A) hMSCs uptake of BMEVs labeled with PKH67 dye (green) after 24 h of treatment at 37 °C or 4 °C (control). (B) Representative pictures of cells stained with Alizarin red after BMEVs treatment (200 µg/ml) (25×). Quantification of (C) mineralization by Alizarin red staining and (D) cell proliferation by crystal violet staining in hMSCs that received different concentrations of BMEVs (2, 20 and 200 µg/ml). (E) XTT assay measured after 4, 8 and 24 h of substrate addition. Collagen production is represented by (F) photomicrographs of Picrosirius Red/Fast green staining (100×), (G) ratio of collagen/cells determined by Picrosirius red/crystal violet staining and (H) mRNA expression of *col1α1* and *col1α2*. (I) Alkaline phosphatase enzymatic activity. (J) mRNA expression of *RUNX2*, *OSX*, *ALP*, *OC*, *OPN*, *RANKL*, *OPG*, *TGF-B1*, *TGF-B2*, *BMP-2*, *FGF-2*, *WISP-1* and *AXIN2* of hMSCs in the presence of osteogenic medium and BMEVs (200 µg/ml) ($n=4$). Bars represent the mean ± S.E.M. $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$ *vs.* control.

showed that miR-29a was present in BMEVs (Supplementary Fig. 2). Thus, these data indicate that BMEVs have the potential to alter osteoblast differentiation and consequently bone formation *via* uptake or extracellular interaction.

4. Discussion

Bone formation is frequently associated with milk consumption, acting as a regulator of bone remodeling in the health and disease state


A
Control
BMEVs
4 days
7 days
14 days
21 days
28 days
B
Alizarin Red (A590 nm) % control
Control
BMEVs
Time (days)
C
Mineralized Nodules (#)
28 days
D
Crystal Violet (A540 nm)
Time (days)
E
Collagen/cells (relative unit)
F
SOST (fold increase)
G
Runx2 (fold increase)
H
Collagen 1α1 (fold increase)
I
Collagen 1α2 (fold increase)
J
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K
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L
FGF-2 (fold increase)
M
WISP-1 (fold increase)

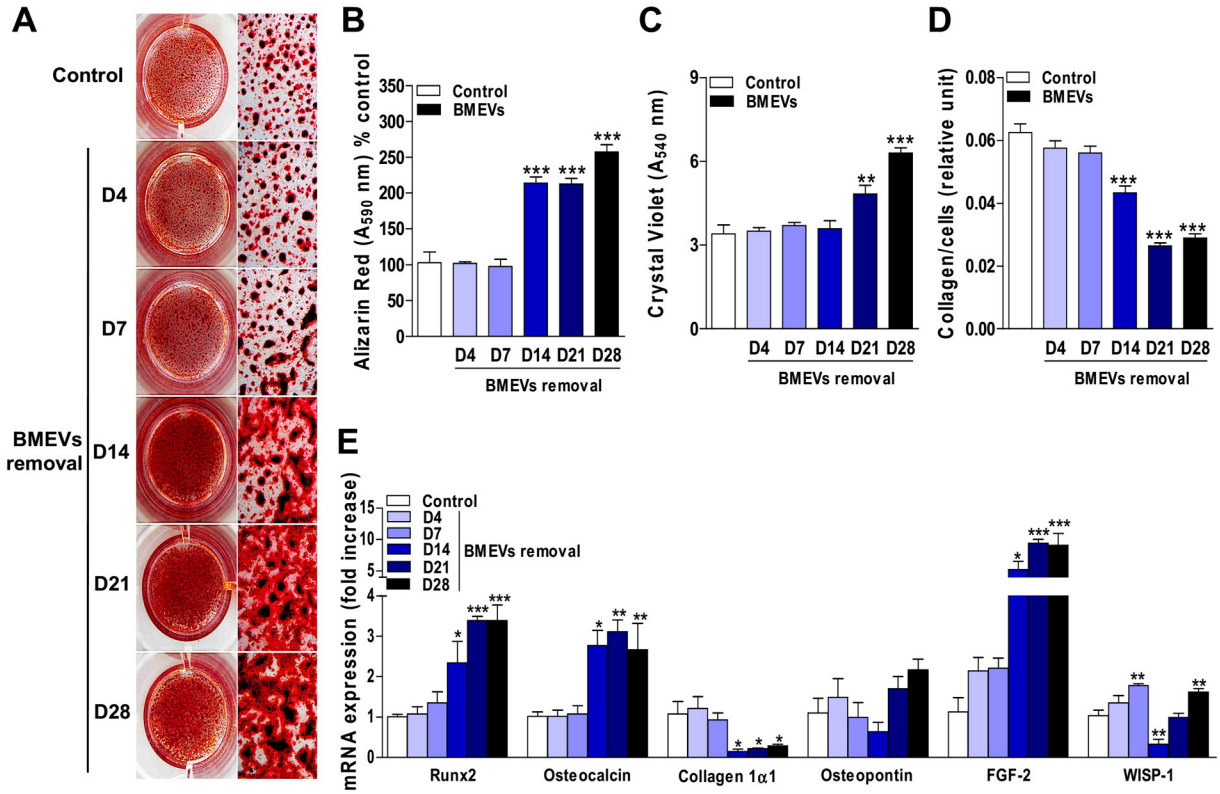

Fig. 4. The effect of duration by removing bovine derived-milk extracellular vesicles (BMEVs) from osteoblast differentiation medium. Data represent time combinations of treatments with BMEVs (200 μg/ml) on hMSCs finishing at day 28. BMEVs were added at day 0 and replaced by only osteogenic medium at the times indicated: day 4, 7, 14 and 21. The control (osteogenic medium) and BMEVs D28 groups were maintained with the same medium composition during all period of treatment. (A) Representative pictures of cells stained by Alizarin red (4× and 25×). Quantification of (B) mineralization by Alizarin red staining and (C) cell proliferation by crystal violet staining. (D) Ratio between collagen and cells determined by Picrosirius red/crystal violet staining. (E) Cells mRNA expression of *RUNX2*, *osteocalcin*, *collagen 1α1*, *osteopontin*, *FGF-2* and *WISP-1* ($n=4$). Bars represent the mean ± S.E.M. $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$ *vs.* control.

[7,8,10–12]. Herein, we have shown the following findings: (a) oral delivery of BMEVs to mice increased osteocytes number in trabecular bone and woven bone formation; (b) human MSCs differentiated into osteoblasts and treated with BMEVs showed increased osteoblast differentiation; (c) during the osteoblast differentiation with BMEVs, the immature phase may appear to predominate; however, the transition to osteocytes was also accelerated; (d) even with increased mineralization, the alteration in the differentiation process by BMEVs led to the impairment of collagen production, related genes and osteonectin as well as mineralized nodule formation (Fig. 6).

During the process of bone formation, the change from calcifying cartilage to trabecular bone passes through the following stages of calcification: calcified cartilagenous matrix, woven bone and, subsequently, lamellar bone. The woven bone is characterized by a tissue rapidly formed, randomly distributed collagen fibrils and with a different composition of extracellular matrix as compared to lamellar bone [27]. It is highly present in bone diseases such as Osteogenesis Imperfecta and Paget's disease of bone and during fracture healing, being considered more fragile [28]. Mice that received BMEVs orally showed an increase in woven bone presence, indicating a rapid bone formation rate as confirmed also by increased trabecular osteocytes number. Indeed, it was already described that the osteocyte population in woven bone is larger than in lamellar bone [29]. In addition, the reduction of adipocytes in the bone marrow also demonstrates that BMEVs lead to a preference of MSCs to differentiate into osteoblasts, as they have the same progenitor [30]. Although all of these alterations were observed even within 7 weeks, it is still important to investigate the effect of BMEVs at long-term on bone formation in the health and disease state.

The development of osteoblasts is regulated by different genes and characterized by stages from preosteoblasts to osteocytes. We demonstrated that *RUNX2*, which is a transcription factor that plays an important role in the osteoblast differentiation, was up-regulated by BMEVs. Interestingly, it also appears that there is a certain period necessary with BMEVs exposure to stimulate and regulate *RUNX2* gene leading to the observed osteoblast response on mineralization, proliferation and collagen deposition. The exposure of BMEVs on MSCs during the first 2 weeks was enough to get a full blown effect of these parameters, and they were also outstanding when cells were treated since the beginning. It was described that overexpression of Runx2 in the early phase of osteoblast development is related to the inhibition of osteoblast maturation with consequent fractures in mice [31]. Normally, a reduction in Runx2 expression leads the maturation of osteoblasts and formation of mature bone [32]. However, this reduction was not observed in the BMEVs group treated during 28 days, indicating the impairment of osteoblast maturation. These results suggest that the exposure to BMEVs in determinate phase of cellular development and how long may alter the bone matrix formation and consequently influence the bone remodeling.

Fig. 3. Kinetic effect of bovine derived-milk extracellular vesicles (BMEVs) on osteoblast differentiation. Data represent BMEVs (200 μg/ml) treatment on human mesenchymal stem cells (hMSCs) for 4, 7, 14, 21 and 28 days in the presence of osteoblast differentiation medium. (A) Representative pictures (25×), (B) quantification of mineralization and (C) number of mineralized nodules/well by Alizarin red staining. (D) Quantification of cell proliferation by crystal violet staining. (E) The ratio of collagen/cells at days 21 and 28 determined by Picrosirius red/crystal violet staining. Expression of osteogenic genes (F) *SOST*, (G) *RUNX2*, (H) *COL1A1*, (I) *COL1A2*, (J) *osteocalcin*, (K) *osteopontin*, (L) *FGF-2* and (M) *WISP-1* in hMSCs induced to differentiate into osteoblast ($n=4$). Bars represent the mean ± S.E.M. $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$ *vs.* control of the respective time.

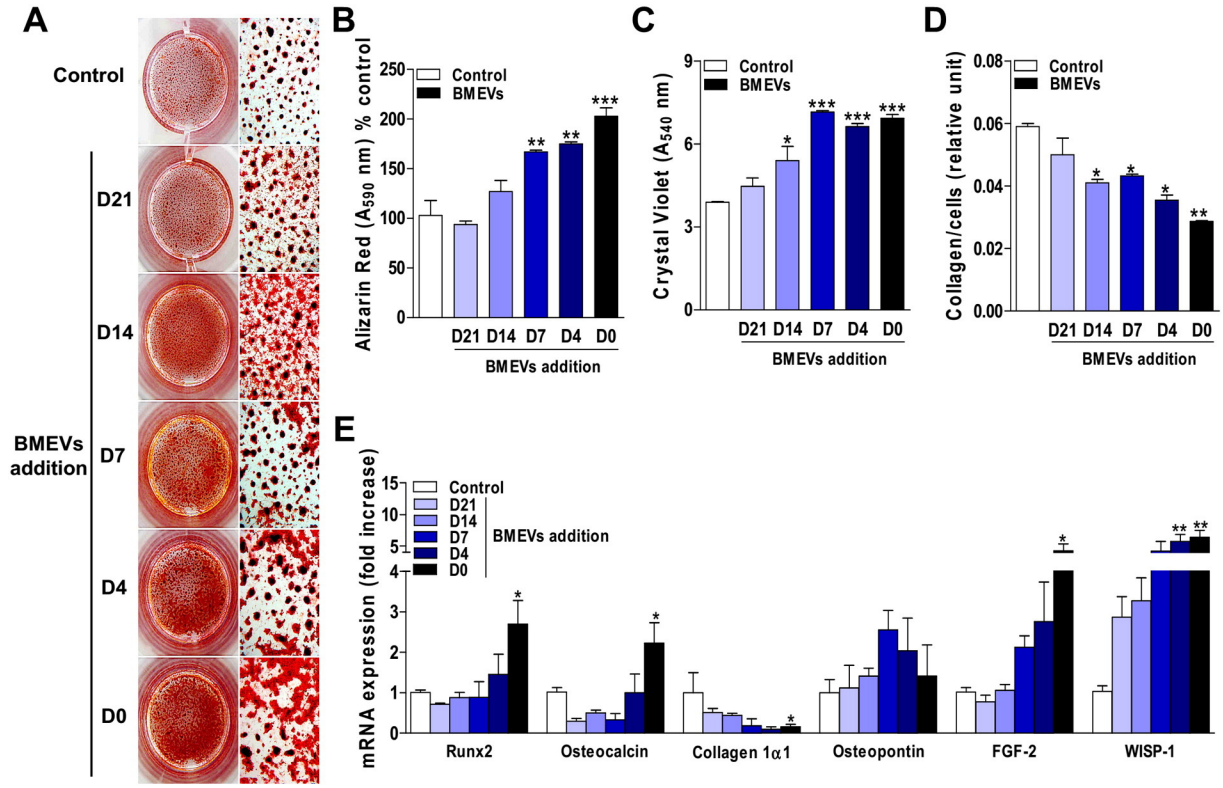

Fig. 5. The effect of duration by adding bovine derived-milk extracellular vesicles (BMEVs) on osteoblast differentiation medium. Data represent time combinations of treatments with BMEVs (200 μg/ml) on hMSCs finishing at day 28. Osteogenic medium was initiated at day 0, and BMEVs were added at the times indicated: day 4, 7, 14 and 21. The control (osteogenic medium) and BMEVs D0 groups were maintained with the same medium composition during all period of treatment. (A) Representative pictures of cells stained with Alizarin red (4× and 25×). Quantification of (B) mineralized by Alizarin red staining and (C) cell proliferation by crystal violet staining. (D) Ratio between collagen and cells determined by Picrosirius red/crystal violet staining. (E) Cells mRNA expression of *RUNX2*, *osteocalcin*, *collagen 1α1*, *osteopontin*, *FGF-2* and *WISP-1* ($n=4$). Bars represent the mean ± S.E.M. $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$ *vs.* control.

Different signaling pathways could regulate Runx2 expression, but BMEVs appear to act mainly by increasing *FGF-2* that is up-regulated in this group. FGF-2 is a growth factor related to cell proliferation [33] and its activation contributes to enhance Runx2 acetylation and stabilization [34]. Activation of Wnt signaling pathway, demonstrated by *WISP-1*, could also induce the expression of *RUNX2* by BMEVs [35].

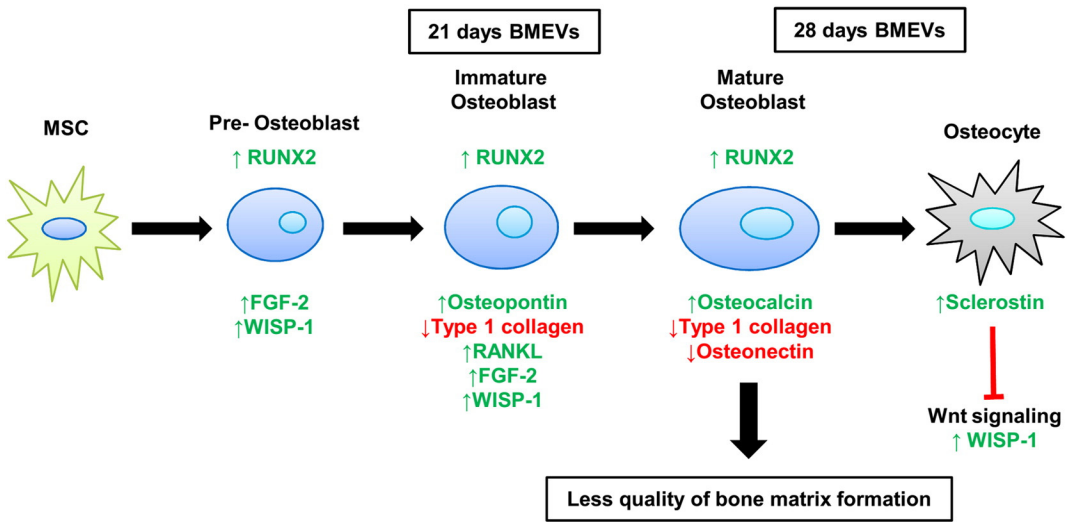

Fig. 6. Effect of bovine derived-milk extracellular vesicles (BMEVs) on osteoblast differentiation. BMEVs contributed with the increase in *FGF-2* and *WISP-1* expression since the beginning of treatment that may maintain the higher expression of *RUNX2* in the cells. This effect leads to the predominance of characteristics related to immature osteoblast phase at day 21. Immature osteoblasts have a higher proliferation rate and may express osteopontin and *RANKL*, leading to immature bone formation and increase in bone turnover. At day 28 is observed an increase in the expression of osteocalcin, which is a marker for mature osteoblast and mineralization, but a reduction in collagen type I production and osteonectin expression. In addition, the increased expression of sclerostin by BMEVs at the same time point may be indicative for osteocytes' presence that appears to be differentiated faster than the control group. The sclerostin may contribute to Wnt signaling inhibition, subsequent osteoclast activation and cessation of mineralization. Altogether, these processes may contribute to the less quality of bone matrix formation.

Little is known about the specific participation of WISP-1 in the osteoblast differentiation; however, studies suggest that WISP-1 increases ALP activity, osteopontin and BMP-2 [36], thereby participating in osteoblast differentiation and proliferation processes. In addition, we demonstrated previously the presence of bioactive TGF-β in BMEVs [15]. Despite invariable *TGF-β* expression in the cells treated with BMEVs, this growth factor could also influence osteoblast differentiation, since it is described to regulate the Runx2 expression, expansion of cells and the bone matrix formation [37,38]. These results indicate that BMEVs may contain factors such as lactoferrin [39] and others (Supplementary Table 2) that increase Runx2 expression, inducing osteoblast differentiation but impede adequate maturation, possibly leading to an impairment of bone matrix formation.

The bone homeostasis process is orchestrated primarily by osteoblasts, osteocytes and osteoclasts. It was described that osteoblasts that escape death *via* apoptosis differentiate into osteocytes and are incorporated within the mineralized matrix [40]. Osteocytes regulate remodeling and bone turnover processes through their dendritic connections, acting on osteoblasts and osteoclasts. Sclerostin is exclusively produced by osteocytes in the bone, and its production implicates in the inhibition of bone formation [41]. The increased expression of sclerostin in BMEVs group at day 28 revealed that osteoblasts became osteocytes faster than the control group. The reason for this could be the enhanced mineralization, since increased sclerostin expression occurs after the onset of *in vitro* mineralization process [41]. Sclerostin is also described to inhibit Wnt signaling pathway, which is related to influence positively bone formation [42], being therefore a target for osteoporotic patients [43]. This effect was also confirmed by lower expression at of *WISP-1* day 28 compared to day 21 in BMEVs group. Furthermore, BMEVs possibly may also influence osteoclasts. The overexpression of Runx2 in mice leads to an elevated bone resorption exceeding the formation by increasing RANKL expression [44]. The expression of RANKL is also related to the differentiation state of human osteoblast, being produced by osteoblasts in the immature phase [45]. Indeed, *RANKL* expression was increased in hMSCs treated with BMEVs at day 21. Based on the evidence presented, BMEVs could potentially inhibit new bone formation by down-regulation of Wnt signaling *via* sclerostin and also enhance bone resorption by osteoclasts *via* increased RANKL expression.

Bone formation is also dependent on the production of collagen and noncollagen proteins. The last one participates in different phases of osteoblast differentiation and activity as well as maturation and mineralization of bone matrix [46]. The mineralization process is regulated by the production of extracellular matrix proteins such as osteocalcin and osteopontin. Osteocalcin is expressed in osteoblast only in the postproliferative phase and during the mineralization process [47]. Indeed, osteocalcin expression was related to the increase in mineralization caused by BMEVs treatment at day 28. Unlike osteocalcin, osteopontin is expressed during the active phase of proliferation [48] as also observed at day 21 with BMEVs treatment. This proliferation is more characteristic of immature osteoblast presence than mature [49]. Moreover, osteopontin is important to regulate bone mineralization by inhibiting it [50], as observed at day 21. These results indicate positively the presence of immature osteoblasts after treatment with BMEVs at day 21, since osteocalcin and osteopontin are also both described at the different stages of osteoblast differentiation [51]. However, later on, it appears that the cells proceed in the differentiation process, arising the osteocytes' presence.

Despite the increased mineralization observed in BMEVs group, the collagen deposition and genes related were reduced in the hMSCs differentiated into osteoblast at day 28. Collagen type I is the most abundant collagen in the connective tissues and has mechanical functions, providing elasticity and bone structure [52]. Measurement of bone mineral density by dual-energy x-ray absorptiometry (DXA) in individuals is not a predictor of bone strength [53]. The quality of bone is also important to maintain the integrity of bone structure. The increased FGF-2 expression could be responsible for *COL1A1* inhibition observed at long-term with BMEVs stimulation, since this was already demonstrated *in vitro* [54]. Moreover, the inadequate production of collagen type I will impair mineralized nodules formation, as collagen is an important component of mineralized structure [55,56]. The imbalance between *COL1A1* and *COL1A2* expression could also lead to an abnormal production of collagen type I, altering the mineralization as observed in osteoarthritic osteoblasts from patients [57]. Our data indicate that there is a change in the composition of the extracellular matrix produced and it is associated to the prevention of osteoblast maturation and accelerate development to osteocytes. We suggest that BMEVs would have a significant impact on bone formation, reducing bone quality and contributing to the development and/or progression of bone diseases such as osteoporosis and osteoarthritis.

We demonstrated previously that BMEVs contain miRNAs that are implicated in the regulation of the immune response [15,16]. Herein, we showed that BMEVs also express miR-29a. The family of miR-29 is known to act as positive regulators of osteoblastogenesis. Low levels of miR-29 contribute to the regulation of bone matrix proteins since it targets RNAs such as *COL1A1*, *COL1A2*, *COL3A1* and osteonectin (*SPARC*) [26,58]. Osteonectin is considered a link protein between mineral and collagen [24]. It was demonstrated that miR-29a down-regulates osteonectin, suppressing its effect on osteoblast differentiation [58]. Collagen expression is also reduced by miR-29a in different diseases [59,60], resulting in less collagen production. Indeed, osteonectin and collagen genes expression were reduced in BMEVs group at day 28. Moreover, Wnt signaling may also induce miR-29a in osteoblast cells that create a positive feedback loop in this pathway [25,58]. We believe that transfer of miR-29a by BMEVs leads to an upregulation of osteoblast differentiation, but later on reduces collagen and osteonectin expression, thereby impairing subsequent bone matrix formation.

In summary, our data showed that oral delivery of bovine derived-milk extracellular vesicles (BMEVs) to mice increased osteocytes number and woven bone formation, contributing to an altered osteoblast differentiation and bone matrix formation. The BMEVs treatment on human bone marrow-derived MSCs increased osteoblast differentiation. However, it appears that BMEVs contribute to a development and predominance of osteoblasts in the immature phase that in a late period differentiate faster into osteocytes. Moreover, the differentiation process appears to influence the bone matrix formation since collagen deposition was lower even with the increased mineralization, and osteonectin expression and nodule formation was also reduced, indicating impairment of bone quality of matrix formed. We suggest that BMEVs, a component still present in commercial milk, mediate changes in the extracellular matrix formation by regulating genes to accelerate the cellular differentiation into osteocytes, contributing to an inability to organize and form an adequate bone matrix.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2015.11.017.

Conflicts of interest

None.

Acknowledgement and authors' contributions

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Chapter 7

Milk-derived nanoparticle fraction promotes the formation of small osteoclasts but reduces bone resorption

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Milk-Derived Nanoparticle Fraction Promotes the Formation of Small Osteoclasts But Reduces Bone Resorption

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The general consensus is that milk promotes bone growth and density because is a source of calcium and contains components that enhance intestinal calcium uptake or directly affect bone metabolism. In this study, we investigated the effect of bovine-derived milk 100,000 g pellet (P100), which contains nanoparticles (<220 nm) including extracellular vesicles, on osteoclast differentiation and bone resorption. Bone marrow-derived osteoclast precursor cells were differentiated into osteoclasts by M-CSF and RANKL (control) and in the presence of milk P100. Milk P100 treatment until day 4 increased the number of TRAP-positive mononuclear cells and small (≤5 nuclei) osteoclasts. The number of large (≥6 nuclei) osteoclasts remained the same. These alterations were associated with increased expression of TRAP, NFATc1, and c-Fos. Cells seeded in a calcium-phosphate coated plate or bone slices showed reduced resorption area when exposed to milk P100 during the differentiation phase and even after osteoclast formation. Interestingly, milk P100 treatment enhanced Cathepsin K expression but reduced Carbonic Anhydrase 2 gene expression. Moreover, intracellular acid production was also decreased by milk P100 treatment. Oral delivery of milk P100 to female DBA1/J mice for 7 weeks did not alter bone area; however, increased osteoclast number and area in tibia without changes in serum RANKL and CTX-I levels. We showed for the first time the effect of milk P100 on osteoclast differentiation both in vitro and in vivo and found that milk P100 increased the formation of small osteoclasts but this does not lead to more bone resorption probably due to reduced acid secretion.

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The physiological homeostasis of bone growth and skeletal remodeling is determined by bone formation and resorption rate (Lerner, 2000). Osteoclasts are the cells responsible for bone resorption during these processes. They are derived from the hematopoietic tissue and are formed by the fusion of mononuclear cells (Lemaire et al., 2004). Alteration of local or systemic factors, such as activation of bone remodeling and recruitment of osteoclasts, may trigger detrimental effects in bone mass (Lemaire et al., 2004), which is involved in bone diseases including osteoporosis, inflammatory-related, for example, osteitis, Paget's disease, and metastatic cancer in bone (Cremers and Garnero, 2006; Cochran, 2008; Ralston, 2008). Bone homeostasis is influenced by diverse factors, including nutrition such as milk (Jackson et al., 2006; Lappe et al., 2008). Although some studies associate the consumption of milk with improved bone density (Sandler et al., 1985; Kalkwarf et al., 2003; Uenishi et al., 2007), others found a positive relationship with bone loss and occurrence of fractures (Bonjour et al., 2008; Michaelsson et al., 2014) or reported no effects in the process of bone remodeling (Feskanich et al., 1997, 2003).

Extracellular vesicles (EVs), which are described to mediate the cellular communication, are also present in milk and by this milk may influence health and disease states (Sun et al., 2013; Wolf et al., 2015). The content of EVs is composed of proteins mRNA and microRNA (miRNA), all being considered as potential modulators of immunity and cell metabolism (Vlassov et al., 2012; Oliveira et al., 2016). Previously, we have shown that nanoparticles derived from bovine milk, which also contain EVs, have immunoregulatory properties on immune cells in vitro (Pieters et al., 2015) and have antirheumatic effects in two mouse models of rheumatoid arthritis (Arntz et al., 2015). Recently, we have also demonstrated the effect of these nanoparticles, which are obtained after 100,000 g ultracentrifugation, on osteoblast differentiation and bone formation in vivo and in vitro by accelerating osteoblast differentiation into osteocytes. However, this was associated

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with an impairment of bone matrix formation (Oliveira et al., 2016).

In this study, we investigated the effect of bovine-derived milk 100,000 g pellet (P100) on osteoclast differentiation and activity by using murine bone marrow-derived osteoclast cells and mice treated orally with milk P100. We demonstrated that milk P100 increased small osteoclast differentiation in vitro and in vivo. However, even with increased differentiation there is an impairment of osteoclast activity that we suggest to be caused by reducing acid secretion.

Materials and Methods

Isolation of the nanoparticle fraction from commercial milk

Bovine-derived milk 100,000 g pellet (P100) was isolated from commercial semi skimmed cow milk as previously described (Arntz et al., 2015; Pieters et al., 2015). In short, milk was centrifuged at 70,000*g* for 1 h at 4°C to remove fat globules, proteins, and other debris. The transparent part of the supernatant was filtered through a Whatman paper nr.1 followed by nr.50. Thereafter the effluent was filtered with a syringe filter (0.22 μm). This effluent was centrifuged at 100,000*g* for 1.5 h at 4°C. The pellet was dissolved in PBS overnight at 4°C on a tube rotary system. The total mixture was centrifuged (10 min, 2,000*g* at 4°C) and the supernatant was sterilized. The amount of protein was measured with a Micro-BCA kit (Thermo Scientific, Pierce, Rockford, IL) and proteomic analysis was previously shown (Arntz et al., 2015). Further characterization was done by Dynamic light scattering (DLS) and EVs had a mean diameter around 100 nm. The amount of EV particles per protein content was $1{,}997 \times 10^{10}$ particles/15 mg of protein in 1 ml. EVs had all the characteristics of exosomes according to the International Society of Extracellular Vesicles (Lotvall et al., 2014).

Cell culture, osteoclast induction, and milk P100 treatment

Bone marrow cells were isolated from femurs of male C57BL/6 mice (10-week-old). The tissue was aseptically removed, and proximal femur was cut off. The bone marrow cavity was flushed out with α-MEM medium (Invitrogen, Carlsbad, CA) using a needle. Bone marrow cells were collected and washed twice with α-MEM. Cells were seeded at an initial density of 100,000 cells in a 96-well plate with α-MEM containing 5% exosome-free FBS, penicillin/streptomycin and 30 ng/ml murine recombinant M-CSF (R&D Systems, Minneapolis, MN). Bone marrow-derived osteoclast precursor cells were plated also in the presence of 2 or 20 ng/ml murine recombinant RANKL (R&D Systems) to stimulate osteoclast differentiation. Cells were incubated at 37°C in a humidified atmosphere. FBS extracellular vesicles-free was obtained after centrifugation at 100,000*g* for 14 h at 4°C with supernatant collection. Cells were treated with milk P100 (200 μg/ml) starting at day 1. The culture medium was replaced every 3–4 days.

Cellular uptake of EVs

EVs were stained with PKH67 Green Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Sigma–Aldrich, St. Louis, MO) as described previously (Pieters et al., 2015). Briefly, PKH67 labeled milk EVs were loaded onto 300 kDa Vivaspin filters (Sartorius Stedim Biotech GmbH, Goettingen, Germany) and centrifuged, washed three times and taken up in culture medium. PKH67 labeled EVs (20 μg/ml) were incubated with bone marrow-derived macrophages or bone marrow-derived osteoclast cells at day 4 of differentiation for 24 h at 37°C with 5% CO_2. After incubation, cells were washed twice, fixed with 4% formaldehyde and stained with 4,′6′-diaminido-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Cellular uptake of EVs was observed using a fluorescent microscope (Leica microscopy, Rijswijk, The Netherlands).

Tartrate-resistant acid phosphatase (TRAP) staining

Cells were washed twice with PBS, fixed for 10 min with 4% paraformaldehyde, and stained for tartrate-resistant acid phosphatase (TRAP) using a leukocyte acid phosphatase cytochemistry kit (Sigma–Aldrich) according to the manufacturer's instructions. The amount of TRAP-positive mononuclear and multinucleated cells was determined. TRAP-positive multinucleated cells containing three or more nuclei under a light microscope were considered to be osteoclast. TRAP-positive multinucleated cells were also stratified according to the nuclei amount as follows: 3–5 nuclei (small), 6–10 nuclei (medium), and >10 nuclei (large osteoclasts).

Resorption pit assay

Calcium phosphate-coated plate was prepared as previously described by Yang et al. (2010). Bone marrow cells were seeded at density of 100,000 cells/well, treated with M-CSF (30 ng/ml) and RANKL (20 ng/ml) on the plate (day 0) and then incubated with milk P100 (200 μg/ml) starting at day 1 or 4. After the incubation period of 7 and 10 days, cells were removed by using sodium hypochlorite bleach and visualized by von Kossa staining. Briefly, cells were stained with 5% aqueous silver nitrate for 30 min at room temperature in the dark. After a washing in distilled water for 5 min the ionic silver was reduced to metallic silver by adding 100 μl of 5% sodium carbonate in 4% formalin and incubated for 4 min at room temperature. The area of resorption was photographed and quantified by using ImageJ software (National Institutes of Health, Bethesda, MD).

Cells were also plated onto 650 μm thick bovine cortical bone slices and treated with osteoclast supplements M-CSF (30 ng/ml) and RANKL (20 ng/ml) for 9 days, adding milk P100 at day 1 or 4. Cells were lysed in water and were mechanically removed by sonification of the bone slices in 10% ammonia for 20 min. The slices were washed and incubated in a 10% saturated alum ($KAl[SO_4]_2\ 12\,H_2O$) solution. Resorption pits were stained with Coomassie brilliant blue (PhastGel Blue R—350; GE Healthcare). Photographs of the resorption were obtained under a light microscope at ×10 magnification, and areas were measured by using ImageJ software (NIH Image).

Cell viability and proliferation assay

Bone marrow cells were seeded at density of 100,000 cells, treated with M-CSF (30 ng/ml) and RANKL (2 or 20 ng/ml) at day 0, and also milk P100 starting at day 1. Cell viability and proliferation were assessed at day 4 using a colorimetric XTT assay (Roche Diagnostics, Indianapolis, IN) according to manufacturer's instructions. The colorimetric change was measured 4 h after addition of substrate and the absorbance was read at 450 nm.

Acridine orange staining

Bone marrow-derived osteoclast cells were treated or not with milk P100, and at day 4 of osteoclast differentiation were incubated with 5 μg/ml acridine orange [3,6-bis(dimethylamine)acridine] for 15 min at 37°C with culture medium supplemented with 20 mmol/L NaCl. The dye was removed by washing in PBS, and then the cells were placed in media, and pictures were taken using a fluorescent microscope 470–490-nm excitation filter and a 520-nm emission filter (Leica microscopy, Rijswijk, The Netherlands). Acidification was measured considering the orange/red area relative to the total area of osteoclast by using ImageJ software (National Institutes of Health).

Quantitative real-time polymerase chain reaction (qRT-PCR)

mRNA was isolated from cells using the TRIzol method (Invitrogen). Isolated RNA was treated with DNaseI (Invitrogen) to remove DNA contamination. Synthesis of cDNA was accomplished by reverse transcription PCR an oligo (dT) primer and Moloney murine leukemia virus Reverse Transcriptase (Applied Biosystems Inc., Foster City, CA). Quantitative real-time PCR was performed using SYBER-Green on a Step-One according to the manufacturer's instructions (Applied Biosystems Inc.). The cycle of threshold (C_t) for each sample was normalized to GAPDH expression. The gene expression was determined by the $2^{-\Delta\Delta Ct}$ method. The primer pair sequences are listed in Table 1.

Mice treatment

Female 10–12-week-old DBA/1J mice (Janvier, Elavage, France) received in the drinking water milk P100 in a particle concentration of 14.3×10^6 EVs/ml during 7 weeks. All mice were housed in filter-top cages, and a standard diet and water were provided ad libitum. The in vivo studies complied with national legislation and were approved by the local authorities on the care and use of animals (DEC-nr: 2014-083).

Histology analysis

Murine proximal tibias were isolated, fixed in 4% phosphate buffered formalin (pH 7.4) and decalcified in 5% formic acid. Tibias were dehydrated with an automated tissue processing apparatus (LeicaASP300, Rijswijk, The Netherlands), embedded in paraffin and sections of 7 μm thickness were obtained. For histology analysis, the sections were stained with TRAP according to the manufacturer's instructions. It was determined the total bone area over tissue area, trabecular bone volume per tissue volume (BV/TV), cortical thickness, osteoclast surface area over bone surface area (Oc.S/BS), and osteoclast number over bone surface area (N.Oc/BS) in the proximal epiphysis and metaphysis of the tibia of PBS and milk P100-treated mice by using Adobe Photoshop C6 software (Adobe Systems Incorporated, San Jose, CA) and ImageJ software (NIH Image).

Serum assays

RANKL was determined in the serum of PBS and milk P100-treated mice by using Quantikine ELISA development kits (R&D System, Inc., Minneapolis, MN) according to the manufacturer's instructions. The marker of bone resorption CTX-I was measured in the serum of mice with the RatLaps™ (CTX-I) EIA assay (Immunodiagnostic Systems Limited, Frankfurt am Main, Germany) according to the manufacturer's instructions.

TABLE 1. Quantitative real-time PCR primer sequence

Gene		Primer sequence (5′- 3′)
TRAP	Forward	GACAAGAGGTTCCAGGAGACC
	Reverse	GGGCTGGGGAAGTTCCAG
NFAT-c1	Forward	ATGCGAGCCATCATCGA
	Reverse	TGGGATGTGAACTCGGAAGAC
c-Fos	Forward	AGGGAACGGAATAAGATGGCT
	Reverse	TCTGTCTCCGCTTGGAGTGT
DC-STAMP	Forward	TGTATCGGCTCATCTCCTCCAT
	Reverse	GACTCCTTGGGTTCCTTGCTT
Cathepsin K	Forward	GAAGCAGTATAACAGCAAGGTGGAT
	Reverse	TGTCTCCCAAGTGGTTCATGG
Carbonic anhydrase 2	Forward	GCTGCAGAGCTTCACTTGGT
	Reverse	AAACAGCCAATCCATCCGGT
CIC-7	Forward	AGCCTGGACTATGACAACAGC
	Reverse	GGAAAGCCGTGTGGTTGATT
CTR	Forward	CGTTCTTTATTACCTGGCTCTTGTG
	Reverse	TCTGGCAGCTAAGGTTCTTGAAA
MMP9	Forward	GGAACTCACACGACATCTTCCA
	Reverse	GAAACTCACACGCCAGAAGAATTT
β3-integrin	Forward	TGGTGCTCAGATGAGACTTTGT
	Reverse	CTGGGAACTCAATAGACTCTGG
Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*)	Forward	GGCAAATTCAACGGCACA
	Reverse	GTTAGTGGGGTCTCGCTCCTG

Statistical analyses

Data are expressed as the mean ± standard error of the mean (SEM). Student's *t*-test was used for comparisons between two groups. Multiple comparisons were performed using one-way ANOVA followed by Dunnett's test in the evaluation of resorption area by osteoclast. The statistical analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA). Statistical significance was stated as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Results

Uptake of EVs and effect of milk P100 on osteoclast differentiation of bone marrow-derived osteoclast precursor cells

As extracellular vesicles can deliver regulating factors into the cell, we first analyzed whether the EVs could be taken up by

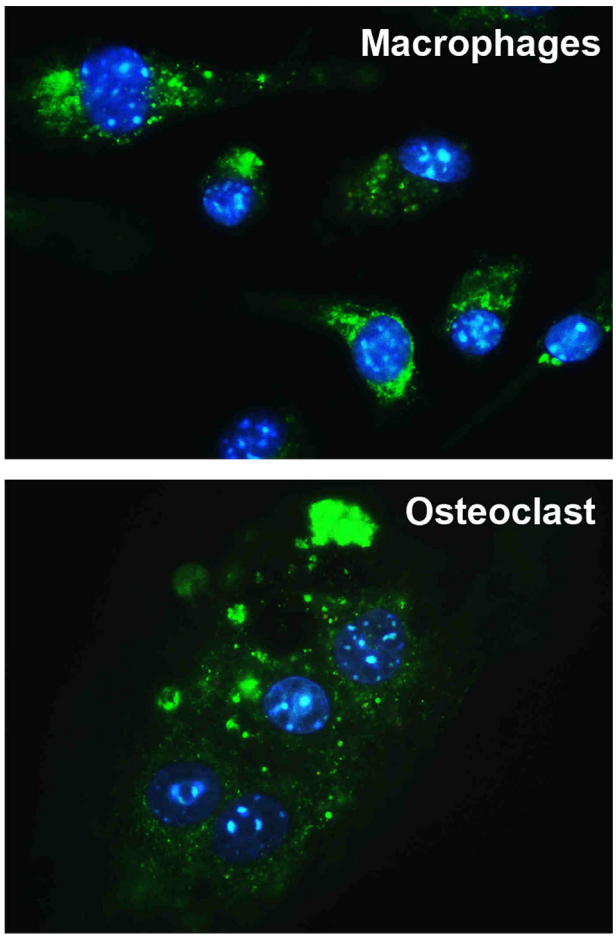

Fig. 1. Uptake of extracellular vesicles (EVs). Uptake of EVs labeled with PKH67 dye after 24 h of exposure by macrophages, (obtained from bone marrow cells after 4 days of 30 ng/ml M-CSF culture), and osteoclasts (obtained from bone marrow cells after 4 days in culture with 30 ng/ml M-CSF and 20 ng/ml RANKL). Labeled EVs are stained in green and nuclei in blue.

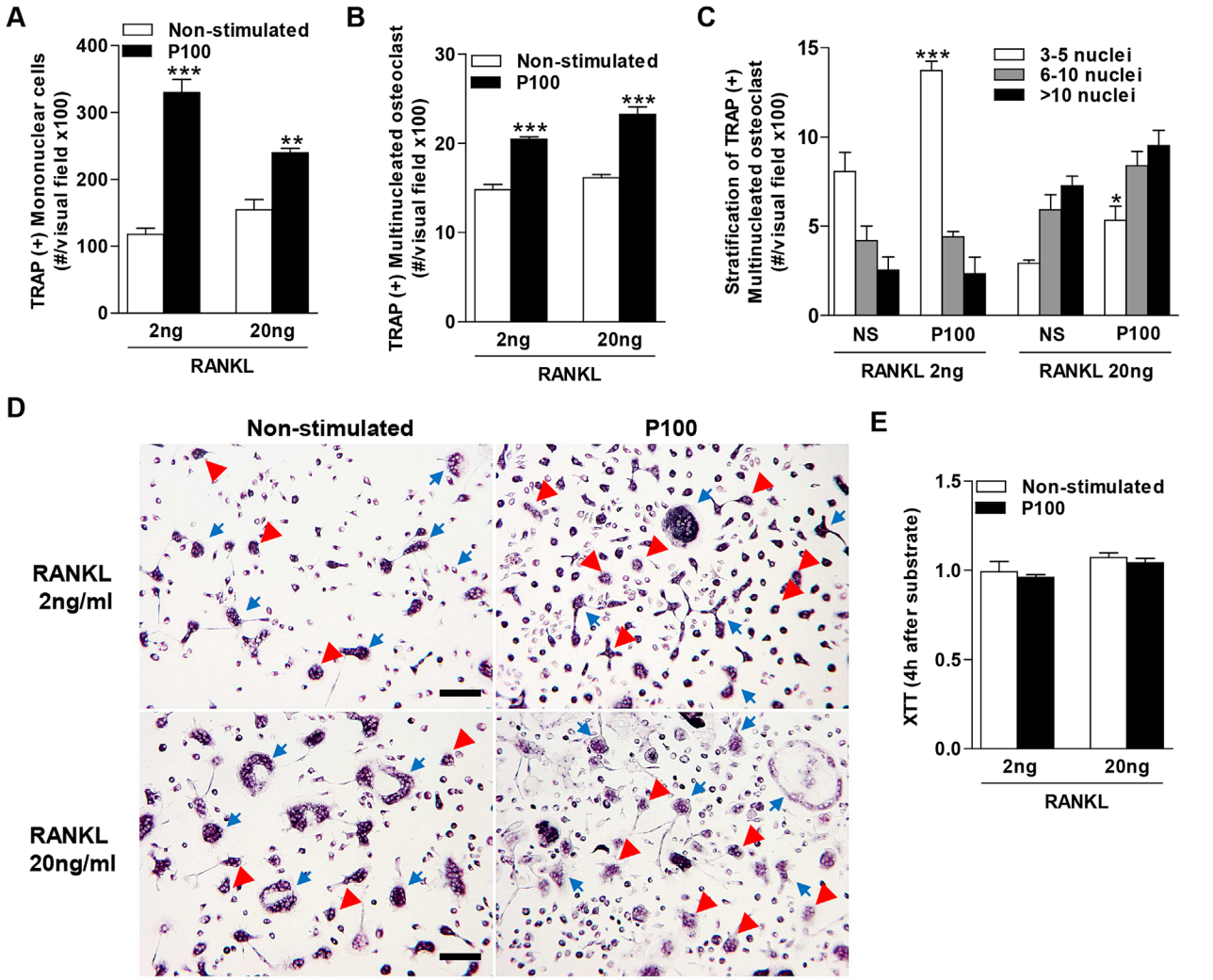

Fig. 2. Effect of bovine derived-milk 100,000 g pellet (P100) on osteoclast differentiation in the presence of M-CSF and soluble RANKL for 4 days. The number of TRAP-positive (A) mononuclear and (B) multinucleated cells (with three or more nuclei). (C) Stratification of osteoclast cells based on the number of nuclei. (D) Representative images of TRAP-positive cells in the culture (×100), arrow heads indicate small TRAP-positive cells and arrows the osteoclast multinucleated cells in the presence of osteoclast differentiation medium and milk P100 (200 μg/ml) added at day 1 (Bars indicate 100 μm). (E) XTT assay measured after 4 h of substrate addition. Bars represent the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus non-stimulated group of respective concentration of RANKL.

osteoclast cells. Osteoclasts at day 4 of differentiation were incubated with fluorescent dye labeled EVs for 24 h. Uptake of EVs by macrophages and osteoclasts was evident (Fig. 1).

The effect of milk P100 on osteoclast differentiation was studied with optimal and suboptimal dose of RANKL. Quantification of TRAP (tartrate-resistant acid phosphatase)-positive cells showed an increase in mononuclear cells (Fig. 2A) and multinucleated osteoclasts (more than 3 nuclei) (Fig. 2B) after treatment with milk P100. When multinucleated osteoclasts were stratified accordingly to nuclei number, it was observed that the treatment with milk P100 increased the number of osteoclast with 3–5 nuclei in both concentrations of RANKL (Fig. 2C). Figure 2D shows representative images of TRAP-positive cells. As the increased number of TRAP-positive cells in milk P100 group could be due to enhanced proliferation of cells, an XTT assay was performed, showing no alteration in the groups evaluated (Fig. 2E).

The expression of genes related to osteoclast differentiation (TRAP, c-Fos, and NFATc1) and fusion (DC-STAMP) treated with milk P100 was also altered after 4 days of culture (Fig. 3). The expression of TRAP is associated with the activation and differentiation of osteoclasts. TRAP was increased in milk P100 group compared with control treated with RANKL 20 ng/ml (Fig. 3A). c-Fos, a component of activator protein 1 (AP-1) and a transcription factor essential for osteoclastogenesis, showed an increase in the expression when cells were treated with milk P100 in both concentrations of RANKL (Fig. 3B). NFATc1 plays the role of a master transcription regulator of osteoclast differentiation; however, differently from c-Fos. Milk P100-treated cells showed an increase in NFAT-c1 only when treated with RANKL 20 ng/ml (Fig. 3C). DC-STAMP is essential for cell–cell fusion in osteoclasts, its expression was reduced only in milk P100 group supplemented with RANKL 2 ng/ml (Fig. 3D).

Effect of milk P100 treatment on osteoclast activity

The main function of osteoclasts is resorb bone. β3-integrin is important for osteoclast migration and formation of the sealing

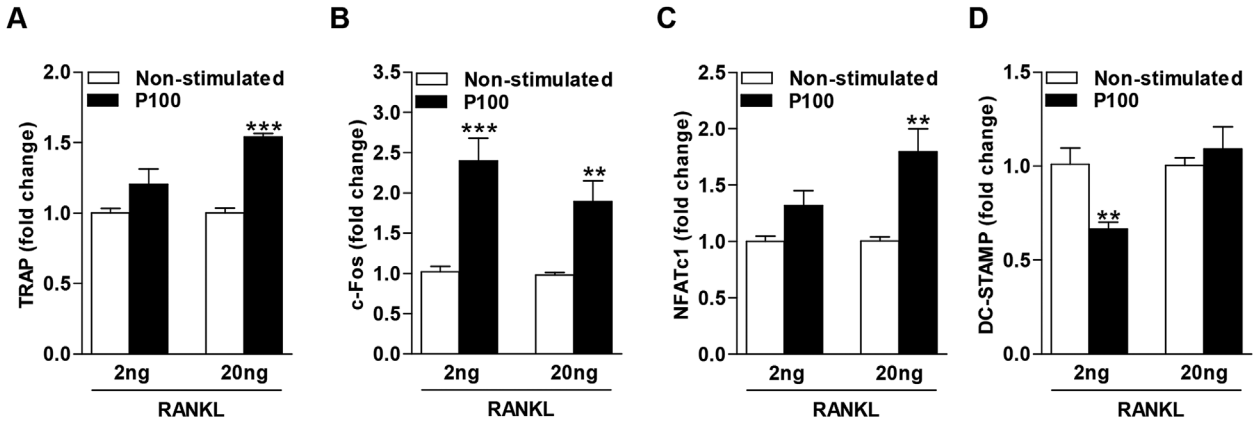

Fig. 3. Expression of osteoclast differentiation genes in bone marrow-derived osteoclast precursor cells treated with bovine derived-milk 100,000 g pellet (P100) at day 4. mRNA expression of (A) *TRAP*, (B) *NFATc1*, (C) *c-Fos*, and (D) *DC-STAMP* in the presence of osteoclast differentiation medium and milk P100 (200 μg/ml) added at day 1. Bars represent the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus non-stimulated group of respective concentration of RANKL.

zone to resorb bone. Its expression was increased in milk P100-treated group 20 ng RANKL (Fig. 4A). The ability of osteoclast to resorb was tested on both calcium-phosphate coated plate, which contains only the inorganic components of bone matrix, and also on bone slices. Although an increase in TRAP-positive cells was observed in the milk P100 group, milk P100 treatment starting at day 1 markedly reduced resorption when cells were seeded in a calcium-phosphate coated plate after 7 and 10 days (Fig. 4B). Milk P100 exposure started at day 4, when osteoclasts were mostly formed, still diminished resorption at both the times evaluated (Fig. 4B). The same reduction of resorption by milk P100 was also observed in bone slices at day 9 (Fig. 4C). Figure 4D shows representative images of the resorption pits in both substrates.

The effect on milk P100 on the expression of genes related to the bone resorption process was studied in more detail (Fig. 5A–E). Cathepsin K, which is responsible for degradation of the organic bone matrix and expressed by osteoclasts, was increased in milk P100-treated group with RANKL 20 ng/ml compared with its control (Fig. 5A). No differences were observed between the groups for CIC-7 expression, a chloride channel, calcitonin receptor (CTR), and matrix metallopeptidase 9 (MMP9) (Fig. 5B–D). However, carbonic anhydrase 2, which catalyzes the reversible hydration of carbon dioxide to be released as hydrogen ions by osteoclasts, was reduced in milk P100-treated group at both RANKL concentrations (Fig. 5E). In order to better evaluate the intracellular acid production in cells treated with milk P100, acridine orange staining in osteoclasts was performed (Fig. 5F). Milk P100-treated cells showed a reduction of acidification compared with control (Fig. 5G).

In order to test whether the effects observed on osteoclasts were caused by milk P100, which contains nanoparticles (<220 nm) such as EVs (exosomes 30–100 nm and micro particles >100 nm) and lipoprotein particles (30–80 nm VLDL, 18–25 nm LDL, 5–12 nm HDL), or pure EVs sized 70–220 nm, we purified the EV fraction using size exclusion chromatography (Supplementary Online Material). As expected, after 4 days of osteoclast differentiation the milk P100-treated cells increased the expression of c-Fos and reduced carbonic anhydrase 2. However, pure EVs were not able to change any of these genes (Supplementary Fig. S1).

Effect of oral treatment with milk P100 on mice

Mice were treated orally with milk P100 for a period of 7 weeks. No alterations in total area of bone, trabecular volume, and cortical thickness were observed (6A–C). In relation to osteoclast analyses, mice treated orally with milk P100 showed an increase in TRAP-positive cells on bone surface in their tibia as compared to control PBS mice as well as osteoclast number (Fig. 6D–F). However, RANKL (Fig. 6G) and CTX-I (Fig. 6H) serum levels, which are considered to be markers for bone resorption, were not different between both groups.

Discussion

Milk consumption is often related to bone remodeling by regulating the processes of bone formation and resorption (Sandler et al., 1985; Feskanich et al., 1997, 2003; Kalkwarf et al., 2003; Uenishi et al., 2007; Bonjour et al., 2008; Gabusi et al., 2012; Michaelsson et al., 2014). Herein, we have shown that milk P100 treatment increases osteoclast differentiation; however, the resorption of these cells is impaired which might be related to a reduction in acid secretion.

Osteoclast development is regulated by different genes and characterized by gradual differentiation from pre-osteoclasts to multinucleated osteoclasts. The transcription factors NFATc1 and c-Fos are the main regulators of osteoclast differentiation and their expression was increased after milk P100 treatment. The increased expression of these genes could be related to the increase in mononuclear precursors and small osteoclasts by milk P100, since they are up-regulated at an early stage of osteoclast differentiation (Boyce, 2013). In addition, the lower DC-STAMP expression may explain why they do not form large osteoclasts in the 2 ng RANKL group. DC-STAMP is upregulated by NFATc1 (Kim et al., 2008) and mainly produced by multinucleated osteoclasts (Boyce, 2013). Since NFATc1 was increased in milk P100 group treated with 20 ng RANKL, we believe that mononuclear and small TRAP-positive cells contributed also to the unchanged DC-STAMP expression observed.

In the process of bone resorption both the inorganic and organic components are degraded (Blair et al., 1986). We showed an inhibition of osteoclast activity in the groups treated

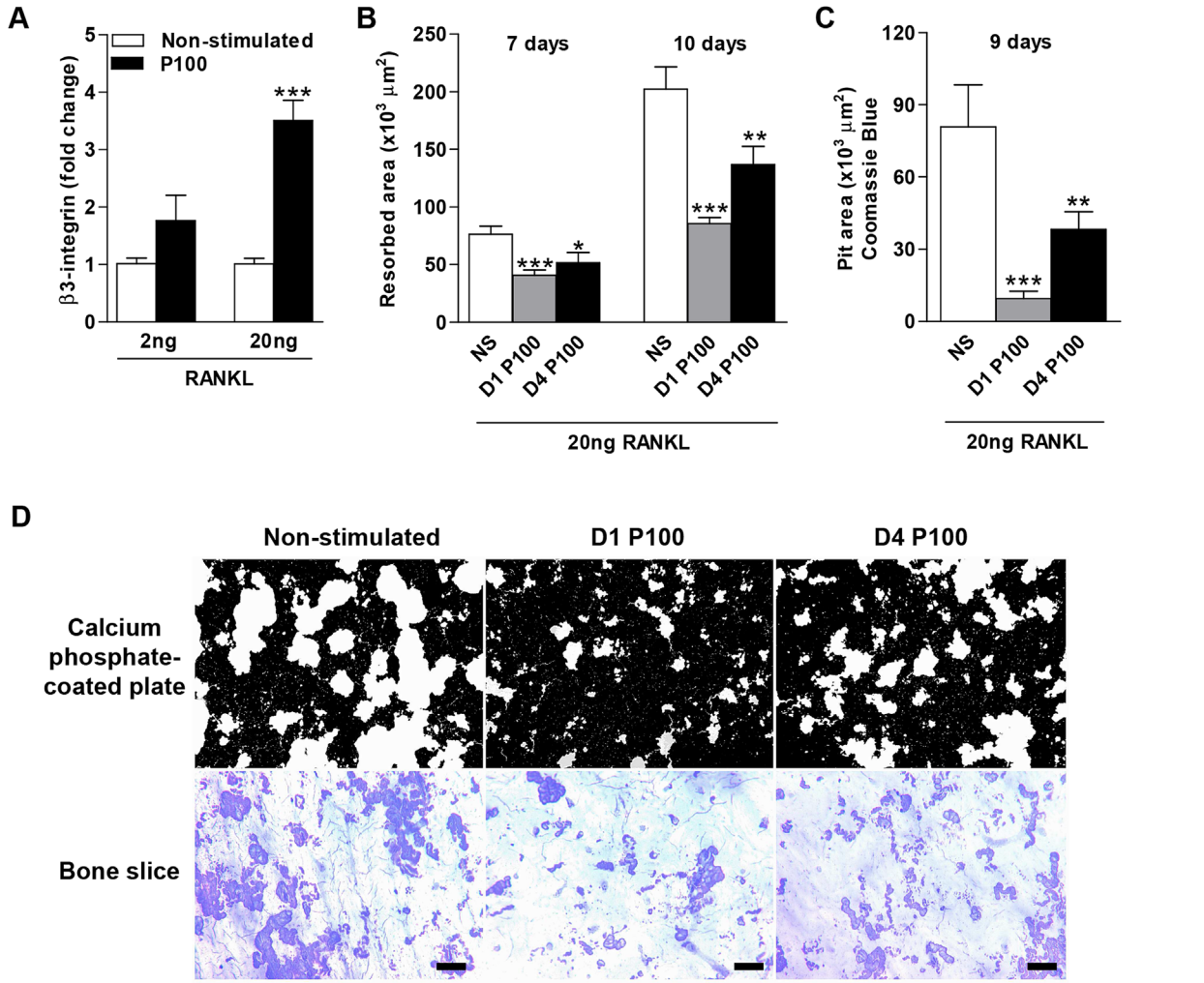

Fig. 4. Effect of bovine derived-milk 100,000 g pellet (P100) on osteoclast activity in the presence of M-CSF and soluble RANKL. (A) mRNA expression of *β3-integrin* in the presence of osteoclast differentiation medium and milk P100 (200 μg/ml). Bars represent the mean ± SEM. $^{***}P < 0.001$ versus non-stimulated group of respective concentration of RANKL. (B) Quantification of resorption area after 7 and 10 days in a calcium phosphate-coated plate and (C) quantification of resorption pit area generated in vitro by osteoclasts after 9 days in bone slice surface. (D) Representative images of calcium phosphate-coated plate and bone slice resorption area (×100) in the presence of osteoclast differentiation medium and milk P100 (200 μg/ml) added at day 1 (D1) or 4 (D4) (Bars indicate 100 μm). Bars represent the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus non-stimulated group.

with milk P100 added at day 1 or 4. These results indicate that independent of the development phase, milk P100 can affect osteoclast activity. The first components of bone matrix to be degraded are minerals, which is dependent on acid release (Teitelbaum, 2000) and changes of pH can significantly influence bone resorption (Arnett and Dempster, 1986). The size of osteoclast may control its acid production and activity. It was described that most small osteoclasts (95%) and also some larger ones (60%) when dependent of Na^+ for acid production are in an inactive or non-resorbing state (Lees and Heersche, 2000), changing the resorption activity (Lees et al., 2001). Then, reduced osteoclast resorption by milk P100 could be due to the presence of small osteoclasts in this group. Carbonic Anhydrase 2 is an important enzyme for the pH regulation and its deficiency leads to osteopetrosis in humans (Sly et al., 1985). We also performed experiments in bone slices that contain not only the inorganic part of bone, but also the organic part and the main constituent is collagen. Even in bone slices a reduction in resorption by milk P100 was noticeable. The degradation of inorganic components of bone precedes the degradation of bone matrix (Blair et al., 1986), indicating that inorganic degradation could be more compromised by milk P100 treatment. Since there was an increase in Cathepsin K expression in milk P100 group, it appears that the medium did not favor its degradation activity of collagen. The enhanced expression of this enzyme in the 20 ng RANKL group treated with milk P100 can be explained by the increased NFATc1 expression in this group (Matsumoto et al., 2004). However, it is known that the enzyme activity of Cathepsin K is higher in acid conditions (Maciewicz and Etherington, 1988). Therefore, it appears that milk P100 can influence osteoclast resorption through alteration in acid secretion.

The process of bone resorption is orchestrated by a combination of not only osteoclast cells, but also osteoblast and osteocytes. Osteoblasts, which are responsible for bone formation, differentiate into osteocytes. When incorporated within the mineralized matrix regulate remodeling and bone turnover processes, acting on osteoblasts and osteoclasts

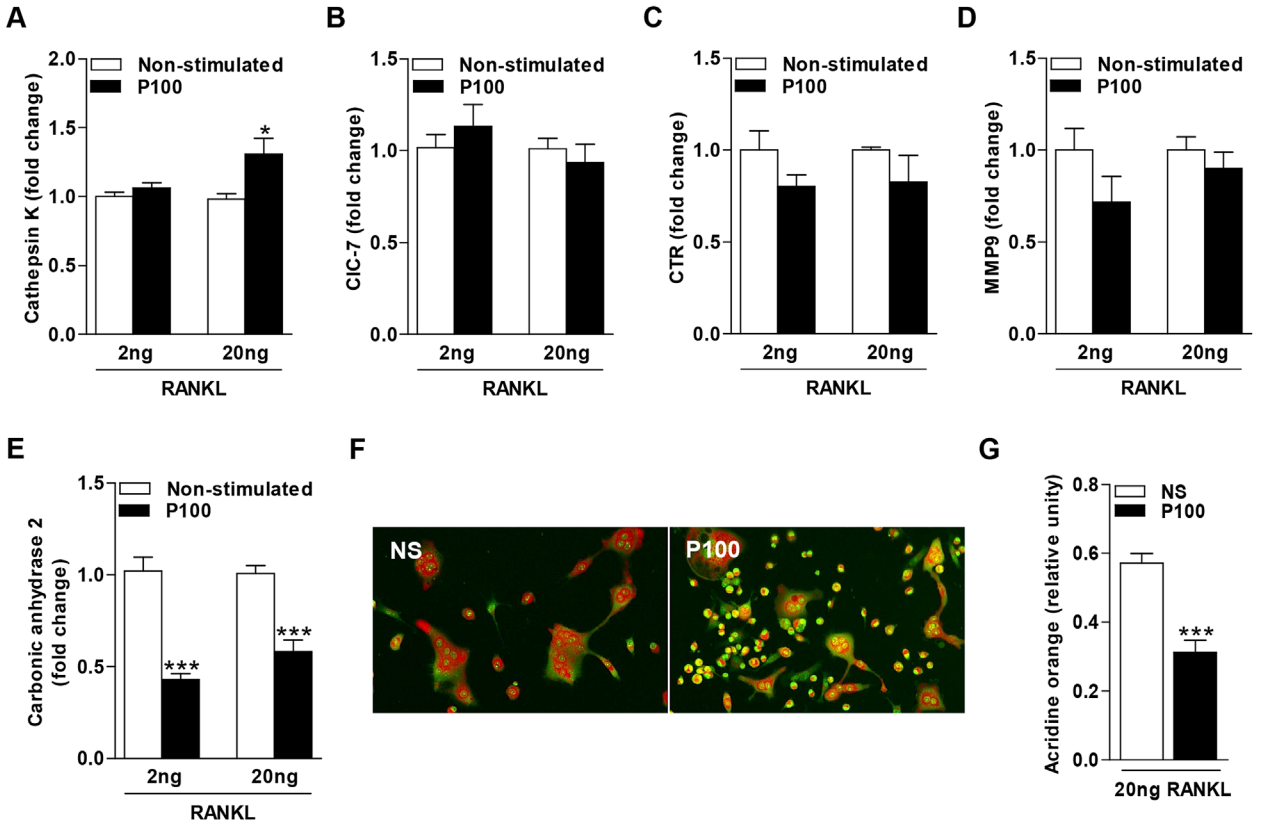

Fig. 5. Gene analyses of markers for osteoclast activity in bone marrow-derived osteoclast precursor cells treated with bovine derived-milk 100,000 g pellet (P100). mRNA expression of (A) *Cathepsin-K*, (B) *CIC-7*, (C) *Calcitonin receptor (CTR)*, (D) *Matrix metallopeptidase (MMP9)*, and (E) *Carbonic Anhydrase 2* in the presence of osteoclast differentiation medium and milk P100 (200 μg/ml). Bars represent the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus non-stimulated group of respective concentration of RANKL. (F) Representative images of acridine orange, acid compartments stain in red/orange and cell nuclei stain in green at day 4 in the presence of osteoclast differentiation medium and milk P100 (200 μg/ml) added at day 1. (G) Quantification of intracellular acid production by acridine orange measured considering the orange/red area relative to the total area of osteoclast. Bars represent the mean ± SEM. $^{***}P < 0.001$ versus non-stimulated (NS) group.

(Manolagas and Parfitt, 2010). We previously demonstrated that mice treated orally with milk P100 showed a bone with more osteocytes cells and was immature (Oliveira et al., 2016). RANKL produced by osteoblasts (Atkins et al., 2003) and osteocytes (Nakashima et al., 2011) can directly enhance bone resorption by osteoclasts. Despite serum RANKL levels were not altered between the groups, its local production by osteocytes may occur as these cells were increased in the tibia by milk P100 (Oliveira et al., 2016). Moreover, milk P100-treated mice did not show alteration in CTX-I levels in the serum, which is a cleavage peptide of collagen type I and a marker for bone degradation (Herrmann and Seibel, 2008). Even though more osteoclast cells could be observed in the tibia, there was no correlation with systemic measurements. Bone analyses were done only in tibia, a long bone, being still necessary to evaluate other bones in the body that are more prone to show altered turnover such as jaw bone and lumbar spine. These experiments were also done in healthy mice, so the effect of milk P100 on bone during a disease could still be different and the fact that milk P100 modulates both osteoblast and osteoclast differentiation warrants further research.

We previously showed that the presence of miRNAs and proteins in milk P100 influences the immune response (Arntz et al., 2015; Pieters et al., 2015) and bone formation (Oliveira et al., 2016). Despite pure EV fraction could not alter genes related to osteoclast differentiation and activity, we still believe that these particles have an effect, possible related to smaller vesicles (exosomes) or in combination with chaperon proteins such as milk lactadherin (MFG-E8) that bridges via its Arg–Gly–Asp sequence to αvβ3 and αvβ5 expressed on the cell membrane and a phosphatidylserine (PS) binding sequence to the PS-containing exosomes (Veron et al., 2005). According to the miRNAs detected before in milk P100, some of them were already described to influence osteoclast regulation, such as miR-29a, -223, and -21 (Sugatani and Hruska, 2007; Franceschetti et al., 2013; Wang et al., 2013). Among them we suggest that miR-21 may be a good candidate to contemplate the major alterations in osteoclast differentiation and activity resulting from treatment with milk P100. This miRNA was described to enhance osteoclast differentiation (Sugatani et al., 2011) and inhibit apoptosis (Sugatani and Hruska, 2013), as observed in milk P100-treated cells. Despite it has not yet been explored in osteoclasts, Chu et al. (2015) showed that the presence of miR-21 reduced the expression of carbonic anhydrase 2 in cancer cells. Interestingly, aplenty of miR-21 has been found in extracellular vesicles derived from milk (Chen et al., 2010). Milk P100 could also contain lipoproteins such as HDL and LDL. Interestingly, osteoclastogenesis from bone marrow cells obtained from LDLR knockout mice showed that LDL has the opposite effect as we found with milk P100, showing that LDL enhances fusion and bone resorption (Okayasu et al., 2012). Moreover, milk P100 also contain

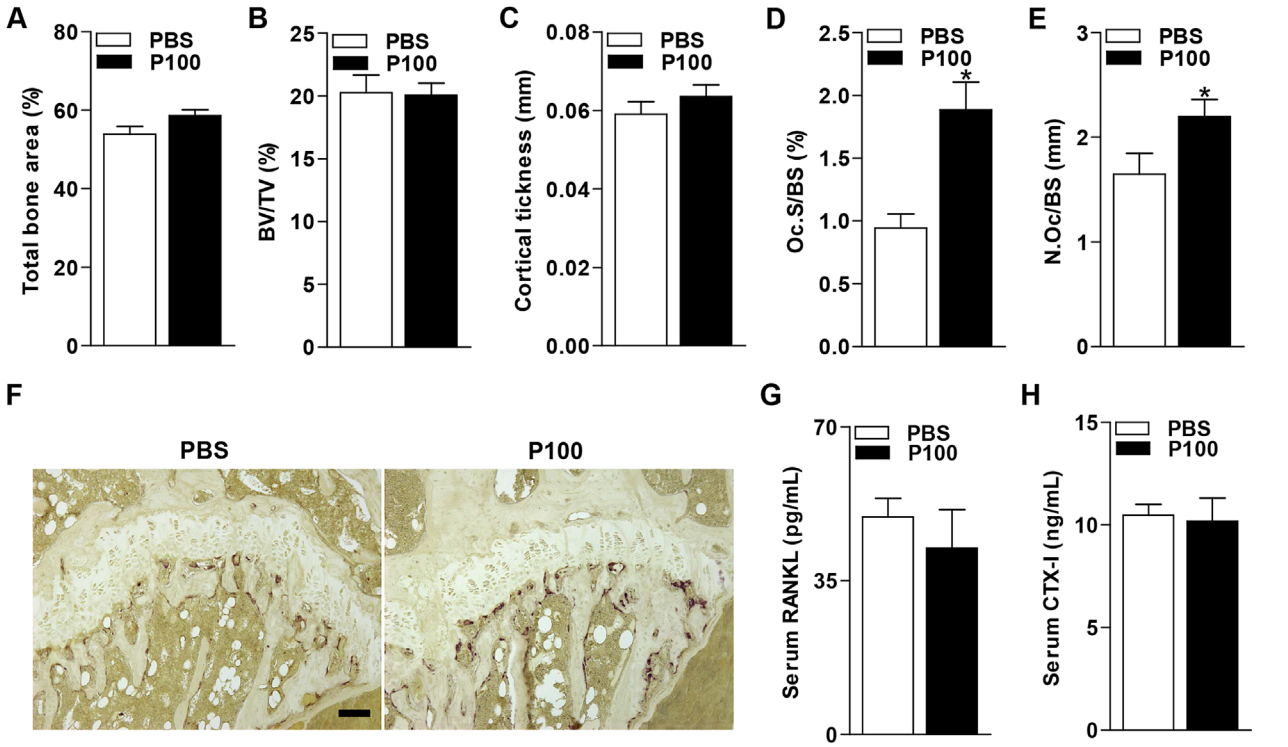

Fig. 6. Tibia alterations after milk P100 exposure in vivo. Mice were treated with bovine derived-milk 100,000 g pellet (P100) in a particle concentration of 14.3×10^6/ml or an equivalent volume of particle buffer (PBS) by oral delivery via drinking water during 7 weeks. (A) Total bone area over tissue area; (B) BV/TV, trabecular bone volume per tissue volume; (C) Cortical thickness; (D) Oc.S/BS, osteoclast surface area over bone surface area; (E) N.Oc/BS, osteoclast number over bone surface area; (F) Representative histology of proximal tibia stained with TRAP (100×), bar indicates 100 μm. Serum (G) RANKL and (H) CTX-I levels of PBS and milk P100-treated mice. Bars represent the mean ± SEM (n = 5–7). $^{**}P < 0.01$, $^{***}P < 0.001$ versus PBS group.

factors such as the protein lactoferrin (Arntz et al., 2015), which is described to reduce osteoclast resorption (Lorget et al., 2002; Yamano et al., 2010), influencing the osteoclast activity. Another hypothesis is related to modulation of microbiota. Some works demonstrated that nutrients can alter microbiota, act indirectly in other organs and influence disease state (Daniel et al., 2014; Collins et al., 2015). Then, we suggest that the effect of milk P100 on osteoclasts could be related to miRNA, protein content, as well as microbiota alteration.

Herein, we showed that the treatment with bovine derived-milk 100,000 g pellet (P100) on bone marrow-derived osteoclast precursor cells alters osteoclast differentiation by increasing the number of small osteoclasts. However, milk P100 treatment appears to influence the osteoclast activity through the inhibition of acid production. Our observation in mice is in line with in vitro data. Milk P100 enhance osteoclast formation in the tibia of mice yet the serum RANKL and CTX-I were unchanged. It may well be that the amount, duration and interval of exposure, the composition of milk, health status, and age determine the final outcome of milk on bone in each human individual. We clearly showed that also nanoparticles in milk are bone modulators that regulate both osteoblast and osteoclast differentiation.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Chapter 8

Summary, final considerations and perspectives

Chapter 8: Summary, final considerations and perspectives

The first aim of this thesis was to clarify the impact of acute joint inflammation on metabolism and whether the consumption of diets that change the adipose tissue volume affects the inflammatory response. The second aim was to analyze the effect of bovine-milk extracellular vesicles on chronic arthritis and bone remodeling.

Acute joint inflammation causes metabolic alterations that may also modulate its inflammatory response

Acute inflammation is a consequence of a rapid immune response in order to protect the organism against antigens. Consequently, this response leads to mobilization of metabolic substrates to generate an adequate support for it. In **Chapter 2**, we demonstrated that knee injection of an antigen (mBSA) disrupt metabolic homeostais that were evaluated by analyzing glucose metabolism, lipids (total cholesterol and triglycerides), as well as adipocytokines (adiponectin and leptin). This arthritis model is characterized by acute influx of neutrophils and local production of pro-inflammatory cytokines (Coelho *et al.*, 2008; Sachs *et al.*, 2011). Therefore, we aimed to evaluate whether cytokines or the presence of neutrophils was causing the observed metabolic changes in this model. For that, we used Etanercept, which is commonly used as an anti-TNF drug in arthritic patients (Tam *et al.*, 2007; Daien *et al.*, 2012), and demonstrated an improvement of metabolic alterations. Since a reduction of neutrophils was observed with this drug, we decide to reduce specifically the neutrophil influx into the joint using DF2156A, a CXCR1/2 receptor antagonist. However, DF2156A did not improve the metabolic alterations. Then, we depleted neutrophils to verify whether the presence of circulating neutrophils could still be causing metabolic changes, yet some parameters analyzed were even worsened. Therefore, we considered that TNF may influence metabolic alterations induced by acute joint inflammation, independently of neutrophils presence.

Consumption of diets, mainly rich in carbohydrates and lipids, can alter the adipose tissue mass and induce distinct metabolic alterations. Next, in the same model of acute antigen-induced joint inflammation, we verified how the alteration of adipose tissue remodeling and associated metabolic changes could influence the acute inflammatory response in the knee joint of mice (**Chapter 3**). We found attenuated infiltration of neutrophils in mice fed with high refined carbohydrate-containing (HC)

diet or cafeteria (CAF) diet, which are diets characterized by increasing adipose tissue mass. However, unexpectedly, when mice were supplemented with conjugated linoleic acid (CLA), which is a diet that causes lipodistrophy, we observed similar lower inflammatory response following antigen-challenge. Moreover, hypertrophy of infrapatellar adipocytes in mice fed with HC and CAF diet was observed, and even in those fed with chow diet challenge with mBSA. The alteration in adipocyte area (hypertrophy) was associated with alterations in adipocytokines levels (adiponectin, resistin and leptin) in these groups. On the other hand, mice fed with CLA supplemented diet that demonstrated a reduction in adipose tissue and production of adipocytokines, also presented lower neutrophils presence in the knee joint cavity. Therefore, we reject the possibility that adipose tissue volume is implicated in attenuation of knee inflammation, and we suggest that metabolic alterations, which is a common factor observed in all three diets evaluated, may influence this response.

Chronic arthritis response is attenuated upon the consumption of milk extracellular vesicles

Starting from **Chapter 4**, we shifted our attention to the effect of bovine-milk extracellular vesicles (BMEVs) on chronic arthritis and bone. Extracellular vesicles contain mRNA and miRNA, which are associated with immunoregulatory proprieties. In **Chapter 4**, we showed the effect of oral delivery of BMEVs on IL-1Ra-deficient mice and collagen-induced arthritis model. First, we characterized these vesicles for size, content and origin and also determined some exosomes markers. In the two arthritis models, it was observed macroscopically that BMEV treatment delayed the onset of arthritis, and histology showed diminished cartilage and bone marrow inflammation. BMEV treatment also reduced the serum levels of MCP-1 and IL-6 and their production by splenic cells. BMEV treatment diminished the anticollagen IgG2a levels, which was accompanied by reduced splenic Th1 (Tbet) and Th17 (RORγT) mRNA. These data indicate that BMEVs may have a therapeutic potential in arthritis.

Based on evidences in literature and our results obtained in **Chapter 4**, we hypothesized that BMEVs could be used as tool for therapy of patients with arthritis (**Chapter 5**). We believe that BMEVs may reduce pro-inflammatory mediators and

also attenuate the symptoms of arthritis via their RNA content. Moreover, they could also differentiate T cells into T regulatory cells to suppress the inflammatory process.

Milk extracellular vesicles have an impact on bone remodeling via osteoblast and osteoclast regulation

Milk consumption is highly associated with bone formation, however it is still a controversial issue for having contradictory and inconclusive data (Feskanich *et al.*, 2003; Kalkwarf *et al.*, 2003; Uenishi *et al.*, 2007; Bonjour *et al.*, 2008; Michaelsson *et al.*, 2014). In **Chapter 4,** we found that BMEVs had a profound effect on circulating IL-6 and MCP-1 levels. As both these cytokines are also involved in ostoclast formation and activation, the BMEV treatment could have an indirect effect on bone turnover. In **Chapter 6,** we investigate the effect of BMEVs on osteoblast differentiation *in vivo* and *in vitro*. We provided two different particle concentrations of BMEVs in the drinking water to mice during 7 weeks. Mice that received the highest concentration of BMEVs showed no alterations in trabecular bone area, but an increase in osteocyte count and woven bone formation, and reduction of adipocytes presence in the tibia was also observed. To test whether BMEVs had a direct effect on bone cells we exposed human mesenchymal stem cells during their differentiation process into osteoblasts with BMEVs. In general, BMEVs increased the osteoblast differentiation, with more mineralization and cell proliferation. BMEVs increased the expression of Runx2, osteoprotegerin, RANKL, genes mainly expressed in immature osteoblasts that produce immature bone at day 21. Also, it was observed in the BMEV group an increase in sclerostin expression, which is present only in osteocytes at day 28. Interestingly, the collagen deposition was impaired with BMEV treatment. The fact that osteoblast differentiation is accelerated appears to influence dramatically the bone matrix leading to woven bone formation, which is more brittle and more vulnerable for fracture if not replaced by lamellar bone.

Osteoclasts are cells responsible for bone resorption (Teitelbaum, 2000) and replace the woven bone for lamellar bone. Therefore, we also analyzed the effect of BMEVs on osteoclast differentiation and activity (**Chapter 7**). To do this, we isolated bone marrow cells from mice and differentiated them into osteoclast in a medium supplemented with M-CSF and RANKL, and with or without BMEVs. However, in the last experiments we purified milk extracellular vesicles sized 70-220nm by Sepharose

CL-2B. As this fraction did not show the same results as the non-purified nanoparticles on osteoclasts, we changed the name BMEVs into bovine derived-milk 100,000g pellet (P100). Milk P100 treated group showed an increase in the differentiation of small osteoclasts (3-5 nuclei), and this was associated to increased expression of genes related to the differentiation process, such as TRAP, c-Fos and NFATc1. Then, we analyzed the activity of osteoclasts in a calcium-phosphate coated plate and bone slices. However, on both substrates milk P100 impaired the osteoclast resorbing activity. Genes necessary to osteoclast activity were evaluated to explain this finding. Cathepsin K was increased in milk P100 treated group, however carbonic anhydrase 2, an enzyme responsible for intracellular acid production and bone resorption, was reduced and associated to lower intracellular acid production by osteoclasts. Moreover, mice that received P100 in drink water during 7 weeks showed no alterations in bone area, but increased osteoclast in the tibia associated to unchanged levels of CTX-I and RANKL in the serum. Therefore, although there was an increase in the differentiation of small osteoclasts by milk nanoparticle fraction, their activity was impaired and appeared to be related to a reduction in acid secretion, and our observation in mice was in line with *in vitro* data.

Final considerations and perspectives

Inflammation is a natural defense response of the organism, however in excess can compromise the normal function of body systems. We showed in this thesis that acute joint inflammation in the knee of mice leads to metabolic alterations. The use of inhibitors of cytokines appears to be a better alternative to improve these metabolic changes than targeting the neutrophils. On the other hand, chronic metabolic alterations induced by diet alter the inflammatory response in the knee after challenge with antigen-induced joint inflammation. These two studies demonstrated how the metabolism is functionally linked to the immune system and both of them can influence each other. However, it would be still quite promising to determinate the mechanisms underlying these responses. Differently, in a chronic state of arthritis, we showed that nanoparticles from milk are interesting therapeutic agents to treat arthritis, although their *in vivo* mode of action remains to be elucidated. We believe that extracellular vesicles will become part of the therapeutic armamentarium and further research is warranted whether food-derived EVs can be used for that purpose.

Because milk consumption still occurs after breastfeeding, discussions about the implications on our health in the long run are a matter of debate, being a controversial issue for having contradictory and inconclusive data. The proper identification of the effect of milk components can be very useful in clinical practice, since extracellular vesicles are present in commercial milk even after pasteurization (Pieters *et al.*, 2015). Currently, several strategies have been used to study the role of extracellular vesicles, especially *in vitro*, demonstrating their ability to interact with other cells by modulating their functionality. The presence of extracellular vesicles in milk has instigated researchers to unravel their possible effects, particularly on immunity (Kosaka *et al.*, 2010; Sun *et al.*, 2013). In this thesis, we demonstrated that milk nanoparticles, which contain extracellular vesicles and proteins, exert an important impact on bone remodeling via modulation of osteoblast and osteoclast response. However, additional *in vivo* research is needed to prove that milk consumption has a consequence for bone remodeling and bone disorders. Moreover, the determination of how milk nanoparticle fraction is applying its effect, whether through extracellular vesicles, proteins or a combined effect, is still necessary. The research progress, particularly related to food-disease interaction, is important to create options for therapies to patients who need them. Thus, evaluation of the interaction between bone tissue and its metabolic and inflammatory response by consumption of milk nanoparticles may contribute to increase the knowledge about their effect in physiological processes and bone diseases.

Overall conclusions

In this thesis we have shown that acute inflammation in the knee joint causes metabolic alterations which are mediated by TNF, on the other hand, metabolic alterations appear to alter the joint inflammatory response in the same model after consumption of different diets irrespective of their fat enhancing or reducing effect. In addition, milk nanoparticle fraction exerts a positive effect on two mouse models of chronic arthritis and regulate bone remodeling through osteoblast and osteoclast differentiation, demonstrating to have both an effect on immune and metabolic systems. Taking together, we report how dietary components may influence states of health and disease and this is important to create future strategies of therapies related to arthritis and bone.

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