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Delayed resolution of acute inflammation during zymosan-induced arthritis in leptin-deficient miceEiva Bernotiene^{1,2}, Gaby Palmer¹, Dominique Talabot-Ayer¹, Ildiko Szalay-Quinodoz³, Michel L Aubert⁴ and Cem Gabay¹¹Division of Rheumatology, University Hospital and Department of Pathology, University of Geneva School of Medicine, Geneva, Switzerland²Department of Immunology, Institute of Experimental and Clinical Medicine at Vilnius University, Vilnius, Lithuania³Division of Clinical Pathology, University Hospital, Geneva, Switzerland⁴Division of Development and Growth, Department of Pediatrics, University of Geneva School of Medicine, Geneva, SwitzerlandCorresponding author: Cem Gabay, Cem.Gabay@hcuge.ch

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Arthritis Res Ther 2004, **6**:R256-R263 (DOI 10.1186/ar1174)© 2004 Bernotiene *et al.*; licensee BioMed Central Ltd. This is an Open Access article: verbatim copying and redistribution of this article are permitted in all media for any purpose, provided this notice is preserved along with the article's original URL.**Abstract**

The severity of antigen-induced arthritis (AIA) is decreased in leptin-deficient *ob/ob* mice. However, joint inflammation in AIA depends on the immune response, which is impaired in *ob/ob* mice. In the present study we investigated the effects of leptin deficiency on zymosan-induced arthritis (ZIA), which is independent of adaptive immunity. Arthritis was induced by injection of zymosan into the knee joint. Joint swelling was similar after 6 and 24 hours in *ob/ob* and control mice. However, it remained elevated in *ob/ob* animals on day 3 whereas values normalized in controls. Histology revealed similar articular lesions in all animals on day 3, but on days 14 and 21 arthritis

tended to be more severe in *ob/ob* mice. The acute phase response, reflected by circulating levels of IL-6 and serum amyloid A, was also more pronounced in *ob/ob* mice, although corticosterone was significantly elevated in these animals. Similar results were obtained in leptin receptor-deficient *db/db* mice. Thus, in contrast to AIA, ZIA is not impaired in leptin-deficient animals. On the contrary, resolution of acute inflammation appears to be delayed in the absence of leptin or leptin signalling, suggesting that chronic leptin deficiency interferes with adequate control of the inflammatory response in ZIA.

Keywords: acute phase response, arthritis, inflammation, interleukin-6, leptin**Introduction**

Leptin is a peptide hormone that plays an important role in the regulation of body weight by inhibiting food intake and stimulating energy expenditure. Moreover, leptin exhibits a variety of other effects, including regulation of endocrine function, reproduction and immunity. Consistently, leptin-deficient (*ob/ob*) mice and leptin-receptor deficient (*db/db*) mice are not only obese, but also exhibit important hormonal imbalances, infertility, abnormalities in thermoregulation, and evidence of immune and haematopoietic defects [1-4].

The role of leptin in the modulation of immune response and inflammation has recently become increasingly evident. The increase in leptin production that occurs during infection and inflammation strongly suggests that leptin is a part

of the cytokine cascade, which orchestrates the innate immune response and host defence mechanisms [5]. However, both proinflammatory and anti-inflammatory effects have been described for leptin, depending on the experimental model investigated [5]. Leptin plays an important role in inflammatory processes involving T cells, and has been reported to promote T-helper (Th)1 polarization of the cellular immune response [6-9]. Several studies have implicated leptin in the pathogenesis of autoimmune inflammatory conditions, such as experimental autoimmune encephalomyelitis and type 1 diabetes [10-13].

Furthermore, in leptin-deficient *ob/ob* mice we recently demonstrated reduced severity of antigen-induced arthritis (AIA), which is a model of immune-mediated joint inflammation [14]. Leptin appeared to contribute to the mechanisms

AIA = antigen-induced arthritis; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; IL = interleukin; LPS = lipopolysaccharide; MIF = macrophage migration inhibitory factor; OB-Ra = leptin receptor, long isoform; SAA = serum amyloid A; Th = T-helper (cell); TNF = tumour necrosis factor; ZIA = zymosan-induced arthritis.

of joint inflammation in AIA by regulating both humoral and cell-mediated immune responses. Essentially identical results were obtained in *db/db* mice, which lack expression of the functional leptin receptor, long isoform (OB-Rb). However, joint inflammation in AIA depends on the adaptive immune response, which is known to be impaired in *ob/ob* and *db/db* mice. We conducted the present study to investigate the effect of leptin and leptin receptor deficiency on inflammatory events in the joint, independent of their effects on T-cell and B-cell responses. Therefore, we explored the effect of leptin deficiency in zymosan-induced arthritis (ZIA), a model of proliferative arthritis, which is restricted to the joint injected with zymosan A and is not dependent on the adaptive immune response. Indeed, zymosan A, which is a ligand for toll-like receptor 2 as well as an activator of the alternate complement pathway, triggers local activation of the innate immune system, causing inflammation in the injected joint [15,16].

We followed the development of ZIA in *ob/ob* C57BL/6 mice and in their control *+/?* (i.e. *+/+* or *ob/+*) lean littermates. In addition, in order to evaluate the role of OB-Rb in ZIA, we also used *db/db* and control *db/+* C57BL/KS mice. The results of the experiments show that, in contrast to AIA, ZIA is not impaired in *ob/ob* and *db/db* mice. Furthermore, resolution of acute inflammation during ZIA appears to be delayed in the absence of leptin.

Materials and methods

Animals

Eight-week-old male, leptin deficient C57BL/6 *ob/ob* mice and their control *+/+* or *ob/+* (i.e. *+/?*) littermates, as well as OB-Rb leptin receptor deficient C57BL/KS *db/db* mice and their control *db/+* littermates, were purchased from Elevage Janvier (Le Genest-St-Isle, France). Animals were housed under conventional conditions in the animal facility of the Geneva University School of Medicine. Water and standard laboratory chow were provided *ad libitum*. The experimental protocol received the approval from the Animal Ethics Committee of the Geneva University School of Medicine and of the Geneva Veterinarian Office.

Induction of arthritis

Arthritis was induced by injection of 180 µg zymosan A via a small skin cut along the suprapatellar ligament directly into the knee joint cavity, as described previously [17]. Zymosan A (30 mg) from *Saccharomyces cerevisiae* (Sigma-Aldrich, Buchs, Switzerland) was suspended in 1 ml endotoxin-free saline (Laboratory Dr G Bichsel AG, Interlaken, Switzerland) by boiling and sonification. Mice were injected with 6 µl of this suspension into the right knee joint, under inhalation anaesthesia with 5% isofluran (Forene®; Abbott AG, Baar, Switzerland). The left knee joint was simultaneously injected with an equal amount (6 µl) of saline and served as the control.

Study design

Ob/ob and *+/?* mice were killed at different time points after injection of zymosan A (i.e. on days 1 and 3 for evaluation of the acute phase of arthritis, and on days 14 or 21 for evaluation of the chronic phase). The knee joints were either processed for histology (days 3, 14 and 21) or used for RNA extraction (days 1 and 14). One experiment was performed using *db/db* and control *db/+* mice to evaluate the effect of OB-Rb deficiency on ZIA. In this experiment, mice were killed on day 14 after injection of zymosan A and the knee joints were processed for histology. All animals were killed by exsanguination (cardiac puncture) followed by cervical dislocation, under intraperitoneal anaesthesia with 0.01 ml/g saline solution containing 12 mg/ml ketasol (Dr E Graub AG, Bern, Switzerland) and 0.16% rompun (Bayer, Provet AG, Lyssach, Switzerland).

Isotopic quantification of joint swelling

Joint swelling was quantified at various time points after injection of zymosan A by measuring uptake of circulating ^{99m}Tc-pertechnetate in the knee joint, as previously described [14,17]. Animals were injected subcutaneously in the neck region with 10 µCi of ^{99m}Tc-pertechnetate in 0.2 ml saline. After 30 min mice were sedated by inhalation anaesthesia with 5% isofluran, and accumulation of the isotope due to increased blood flow and oedema in the knee was determined in duplicate by external gamma counting. The ratio between ^{99m}Tc-pertechnetate uptake in the inflamed and that in the contralateral knee joint was calculated. A ratio greater than 1.1 was taken to indicate joint swelling.

Histological studies

Knee joints were fixed in 10% formalin for a minimum of 24 hours and decalcified using 'd-calcifier' (Lerner Laboratories, Pittsburg, PA, USA), containing 14% HCl, over 6–7 hours. They were embedded in paraffin and serial sections of 4 µm were cut for histological analysis. Sections were stained with either haematoxylin–eosin or toluidine blue. Histological assessment was performed in a blinded manner, using an established scoring system for synovial hyperplasia (from 0 = no hyperplasia to 3 = most severe hyperplasia) and inflammatory cell infiltration in the synovium (from 0 = no inflammation to 3 = most severe inflammation). Cartilage damage was determined by toluidine blue staining (from 0 = no change and fully stained cartilage to 3 = total loss of toluidine blue staining and erosions in cartilage). These three scores were added together to obtain a total histological score, as previously described [18]. Only processes taking place inside the joint cavity were taken into account.

Measurement of IL-6, serum amyloid A and corticosterone levels

Blood samples (100 μ l) were taken at baseline and different time points after injection of zymosan A from the tail vein and at the end of experiment by cardiac puncture. Serum levels of IL-6 were measured using a commercial DuoSet ELISA Development System (R&D Systems, Abington, UK). Detection limit for this test is 39 pg/ml. Serum levels of serum amyloid A (SAA) were determined using a direct enzyme-linked immunosorbent assay, as previously described [19]. The detection limit for this test is 13 μ g/ml. Serum corticosterone levels were determined by using a radioimmunoassay (Diagnostic Systems Laboratories, Webster, TX, USA), as previously described [20].

RNAse protection assay

On days 1 and 14 after injection of zymosan A, knee joints from five *ob/ob* and five *+/?* mice were used for RNA isolation. Total RNA was prepared using the TRIzol reagent (Gibco – Life Technologies AG, Basel, Switzerland) according to the manufacturer's instructions. Expression levels of IL-12, IL-10, IL-1 α , IL-1 β , IL-1 receptor antagonist, IL-18, IL-6, interferon- γ , macrophage migration inhibitory factor (MIF), L32 ribosomal protein and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were analyzed by RiboQuant™ RNAse protection assay, using the mCK-2b multiprobe template set from BD Biosciences (Heidelberg, Germany). Briefly, riboprobes were ³²P-labelled and hybridized overnight in solution with 10 μ g total RNA. The hybridized RNA was digested with RNases A and T1, and the remaining RNase-protected probes were purified, resolved on denaturing polyacrylamide gels, and imaged by autoradiography according to the RiboQuant protocol. The protected bands representing cytokine mRNA expression were quantified by phosphor-imaging using a Cyclone Storage Phosphor System (PerkinElmer Life Sciences, Zaventem, Belgium) and normalized for GAPDH expression. Data represent mean \pm SEM ($n = 5$) of values obtained for all detectable cytokines.

Statistical analysis

All data were analyzed using one-way analysis of variance. Data are expressed as means \pm SEM. $P < 0.05$ was considered statistically significant.

Results

Effect of leptin and leptin receptor deficiency on joint swelling in zymosan-induced arthritis

Arthritis was induced in *ob/ob* and control *+/?* C57BL/6 mice by injecting zymosan A into the right knee joint. To assess whether leptin deficiency had an effect on the development of ZIA, we quantified increased blood flow and oedema (which reflect the severity of acute arthritis) by measuring ^{99m}Tc-pertechnetate uptake at various time points after zymosan A injection (Fig. 1a). The ratio

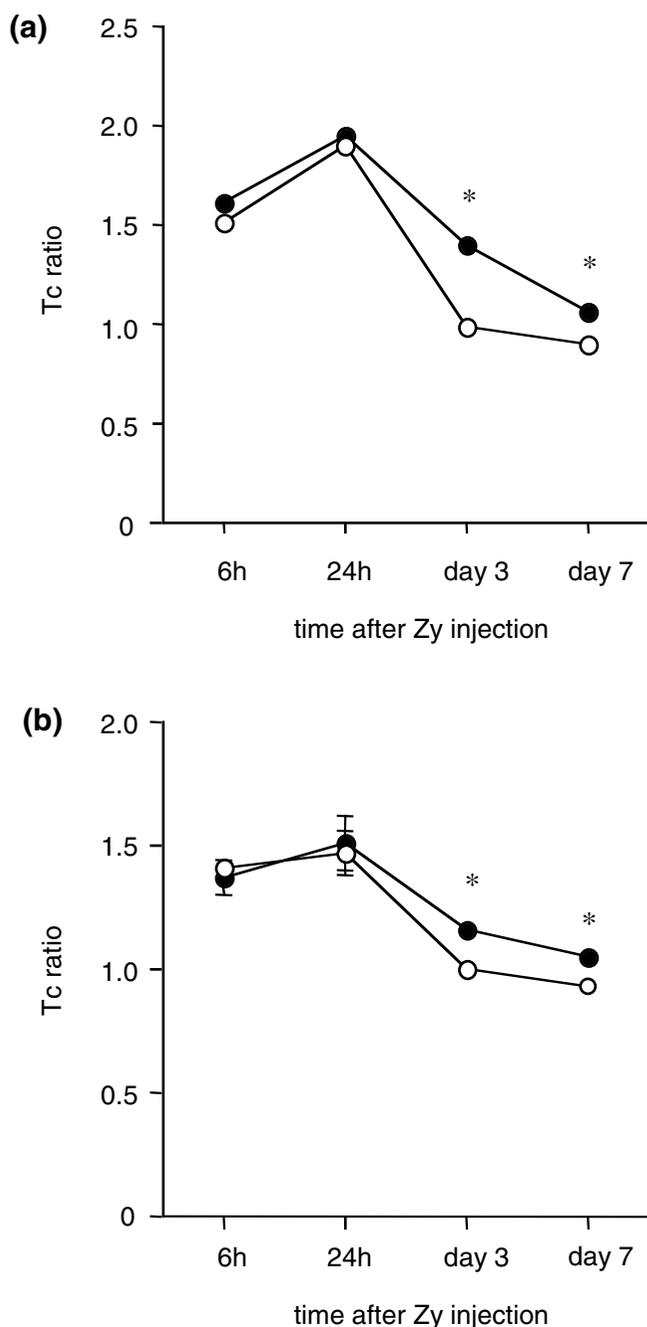
between ^{99m}Tc-pertechnetate uptake in the arthritic joint and that in the control knee increased within 6 hours after injection of zymosan A. They were maximal after 24 hours and declined thereafter. Joint swelling was similar at 6 and 24 hours after injection of zymosan A in *ob/ob* mice and in their *+/?* controls. However, on day 3 ^{99m}Tc-pertechnetate uptake ratios remained elevated in *ob/ob* animals whereas they normalized in *+/?* controls. Resolution of the acute phase of ZIA thus appeared to be delayed in leptin deficient mice. Joint swelling was similar between groups at 7 days (Fig. 1a). In order to investigate whether the observed differences were mediated via OB-Rb, we investigated joint swelling in *db/db* mice and control *db/+* C57BL/KS mice. As for *ob/ob* animals, acute arthritis was similar in *db/db* mice and in *db/+* controls at 6 and 24 hours, but its resolution was delayed and swelling remained detectable on day 3 in *db/db* mice while subsiding in *db/+* animals (Fig. 1b).

Effect of leptin and leptin receptor deficiency on the severity of arthritis

We then investigated the histological features of knee joints from *ob/ob* and *+/?* mice. In both groups overt signs of synovitis were observed in zymosan A injected joints at early (3 days) and late (14 and 21 days) time points after injection (Fig. 2a,2b). In the left control knees (i.e. saline injected) focal accumulation of some synovial cells was observed on day 3 in some of the mice, which was considered to be due to the mechanical injury during intra-articular injection, and no alterations were detected at later stages. On day 3 similar histological changes were observed in zymosan A injected knees of *ob/ob* and *+/?* mice. Both groups exhibited a moderate inflammatory cell infiltration associated with discrete synovial hyperplasia (Fig. 2a,2c). At this early time point cartilage was generally well preserved, as indicated by homogenous toluidine blue staining (data not shown). On day 3 histological scoring repeatedly revealed similar severity of articular lesions in *ob/ob* mice and controls (Fig. 2c). However, in later stages synovial hyperplasia, inflammatory infiltration and cartilage damage were generally greater in *ob/ob* mice than in controls, suggesting a delayed resolution of arthritis in leptin-deficient animals (Fig. 2b,2c). In fact, according to the total histological scores, arthritis always tended to be more severe in *ob/ob* than in *+/?* mice on days 14 and 21, although the differences did not achieve statistical significance (Fig. 2c). This was mostly due to large variation from animal to animal, which was particularly marked in the *ob/ob* group. It is important to note that, at later time points, some very severe cases of arthritis were observed exclusively in the groups of obese animals (Fig. 2b, right panel).

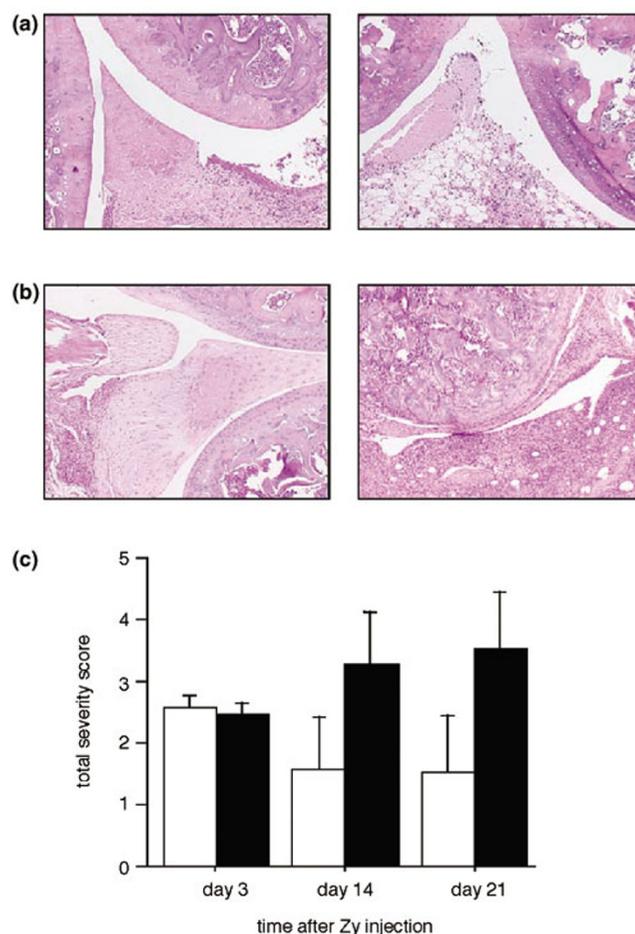
Similar histological features were observed on day 14 in *db/db* mice. Total histological scores tended to be somewhat higher in *db/db* than in *db/+* animals, although again

Figure 1



Knee joint ^{99m}Tc -pertechnetate (Tc) uptake during zymosan-induced arthritis (ZIA). Tc uptake was assessed at different time points after injection of zymosan A in (a) *ob/ob* (black symbols) and *+/?* (white symbols) mice (6 hours, 24 hours, day 3: $n = 9-10$ per group; day 7: $n = 5$ per group), and in (b) *db/db* (black symbols) and *db/+* (white symbols) mice ($n = 6$ per group). Results are expressed as the ratio of Tc uptake in the inflamed to that in the control knee joints. A ratio greater than 1.1 indicates inflammation. In panel (a) data shown for each time point are means \pm SEM in one representative experiment out of five; in panel (b) data shown are means \pm SEM in one experiment. Joint swelling was significantly greater on days 3 and 7 in *ob/ob* as compared with *+/?* mice, and in *db/db* as compared to *db/+* mice ($*P < 0.01$).

Figure 2



Histological changes in the knee joints of *ob/ob* and *+/?* mice during zymosan-induced arthritis (ZIA). (a) Histological changes were examined in zymosan A injected knee joints at an early (day 3) stage of ZIA. Representative sections stained with haematoxylin-eosin are shown for *+/?* (left panel; $\times 100$) and *ob/ob* (right panel; $\times 100$) mice. On day 3 we observed similar articular lesions in both *+/?* and *ob/ob* mice, which exhibited a moderate inflammatory cell infiltration associated with discrete synovial hyperplasia. (b) Histological changes were also examined in zymosan A injected knee joints at a late (day 21) stage of arthritis. A representative section stained with haematoxylin-eosin is shown for *+/?* mice in the left panel ($\times 100$). On day 21 the joints of *+/?* mice exhibited only little inflammation, a discrete reactive synovial hyperplasia and a smooth cartilage surface. At this late stage some very severe cases of arthritis, with pronounced inflammatory cell infiltration and synovial hyperplasia, were observed exclusively in *ob/ob* mice, as illustrated in the right panel (haematoxylin-eosin; $\times 100$). In this example, the articular cartilage has been largely destroyed and overgrown by a hyperplastic and inflammatory synovium. (c) Histological sections were scored for synovial hyperplasia, inflammatory cell infiltration and cartilage destruction. Cumulative total scores are shown for *ob/ob* (black columns) and *+/?* (white columns) mice at early (day 3: $n = 9$ per group) and late (day 14: $n = 9-10$ per group; day 21: $n = 5$ per group) time points after injection of zymosan A, representing histological changes during the early and chronic phases of ZIA, respectively. Data shown for each time point represent means \pm SEM.

Table 1**Serum IL-6, serum amyloid A and corticosterone during zymosan-induced arthritis in *ob/ob* and *+/?* mice**

| Time | IL-6 (pg/ml) | | SAA (μ g/ml) | | Corticosterone (ng/ml) | |
|----------|------------------|---------------------------------|------------------------|-------------------------------|------------------------|-------------------------------|
| | <i>+/?</i> | <i>ob/ob</i> | <i>+/?</i> | <i>ob/ob</i> | <i>+/?</i> | <i>ob/ob</i> |
| Baseline | <39.0 | <39.0 | 21.6 \pm 2.6 | 25.3 \pm 2.5 | 65.6 \pm 15.0 | 140.6 \pm 12.5 ^a |
| 6 hours | 518.0 \pm 52.8 | 1034.1 \pm 114.8 ^a | 331.9 \pm 14.8 | 191.8 \pm 18.7 ^b | 164.5 \pm 15.9 | 216.3 \pm 15.8 ^a |
| 24 hours | 85.7 \pm 25.6 | 154.1 \pm 59.2 | 456 \pm 5 \pm 23.4 | 810.6 \pm 48.3 ^c | 56.9 \pm 8.5 | 149.4 \pm 31.9 ^a |
| 72 hours | <39.0 | <39.0 | 30.9 \pm 4.6 | 387.5 \pm 48.8 ^c | 70.3 \pm 8.4 | 120.6 \pm 19.3 ^a |
| 7 days | ND | ND | 36.4 \pm 2.2 | 184.8 \pm 56.5 ^a | ND | ND |
| 14 days | ND | ND | 131.8 \pm 68.2 | 116.0 \pm 30.1 | ND | ND |

Data shown represent means \pm SEM of five to six mice per group. The results are representative of five independent experiments. Differences between groups were analyzed using one-way analysis of variance. ^a $P < 0.05$; ^b $P < 0.0002$; ^c $P < 0.0001$ for *ob/ob* versus *+/?* mice. ND, not done; SAA, serum amyloid A.

the differences were not statistically significant (total histological severity scores: 5.58 \pm 0.80 for *db/+* mice [$n = 6$] and 6.58 \pm 1.11 for *db/db* mice [$n = 6$]). Leptin and OB-Rb deficiency thus appeared to similarly affect the course of ZIA.

Increased acute phase response in *ob/ob* and *db/db* mice

The acute phase response was examined in *ob/ob* and control mice after injection of zymosan A by measuring circulating levels of IL-6 and of the acute phase protein SAA. In the sera of naïve mice from both groups, IL-6 was undetectable. Following intra-articular injection of zymosan A, serum IL-6 increased within 6 hours in all animals but IL-6 levels were significantly higher in *ob/ob* mice than in controls (Table 1). At 24 hours IL-6 levels were considerably reduced in both groups, and the differences were no longer significant. In *db/db* mice and their lean littermates a similar transient increase in IL-6 was observed, but differences in IL-6 levels between the two groups were not significant (data not shown).

Circulating levels of SAA increased in all animals during the first 3 days after intra-articular zymosan A injection (Table 1). Early after zymosan A injection the increase in circulating SAA was delayed in the *ob/ob* group as compared with *+/?* mice, but later, on days 1 and 3, SAA levels were significantly higher in *ob/ob* animals than in controls. Similar results were obtained in *db/db* and *db/+* mice (data not shown).

Elevated corticosterone levels in leptin and leptin receptor deficient mice

Corticosterone secretion is known to be elevated in all forms of leptin deficiency and in leptin insensitivity [21,22]. During ZIA corticosterone levels increased transiently in both *+/?* and *ob/ob* animals 6 hours after zymosan A injection; however, in the latter group they remained significantly

higher than in *+/?* controls throughout the experiment (Table 1).

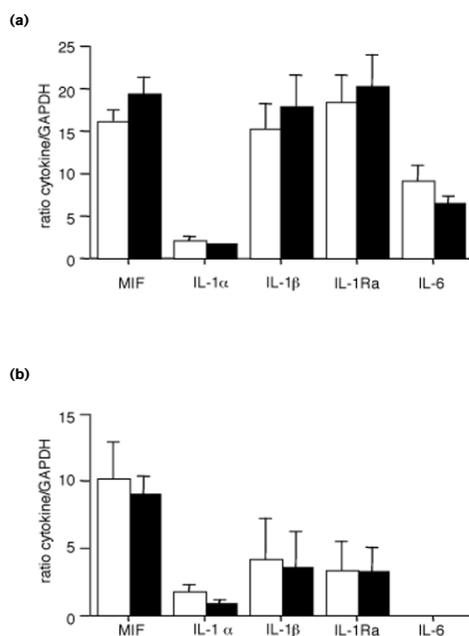
Cytokine mRNA expression in zymosan A injected joints

Expression of mRNAs encoding various cytokines was investigated in knee joints of *ob/ob* and *+/?* animals on days 1 and 14 after zymosan A injection. We observed increased expression of MIF, IL-1 α , IL-1 β , IL-1 receptor antagonist and IL-6 mRNA in zymosan A injected knees (Fig. 3). Furthermore, low levels of IL-18 were also detected on day 14 (data not shown). Expression levels of all of these cytokines were significantly lower or undetectable in the left, saline-injected knee joints in both groups (data not shown). IL-12, IL-10 and interferon- γ were undetectable in both zymosan A and saline injected joints. In contrast to the observed delayed resolution of arthritis and acute phase response in *ob/ob* animals, we could not detect any differences in the local expression of cytokine mRNA in knees injected with zymosan A between *ob/ob* animals and *+/?* controls.

Discussion

The results of the present study indicate that resolution of joint swelling in ZIA was delayed in leptin deficient mice. Accordingly, the acute phase response, as assessed by circulating levels of IL-6 and SAA, remained elevated for a longer period of time in *ob/ob* mice than in control, lean littermates. Furthermore, at late time points histological features of arthritis tended to be more severe in *ob/ob* mice. Similar results were obtained in *db/db* mice, suggesting that the observed changes in the course of ZIA were mediated by a lack of interaction of leptin with OB-Rb.

In contrast to these findings we previously observed a milder form of AIA in *ob/ob* and *db/db* mice as compared with their controls, with decreased synovial levels of IL-1 β and tumour necrosis factor (TNF)- α , and a switch toward production of Th2 cytokines [14]. These contrasting

Figure 3

Cytokine mRNA expression in zymosan A injected knee joints of *ob/ob* and *+/?* mice. Cytokine mRNA expression during acute and chronic phases of zymosan-induced arthritis was investigated on total RNA isolated from the knee joints of five *ob/ob* (black columns) and five *+/?* (white columns) mice on (a) day 1 and (b) day 14 after injection of zymosan A. Cytokine mRNA expression levels were assessed by RNase protection assay (see Materials and methods section), quantified by phosphor-imaging and normalized for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Data are expressed as a ratio of cytokine to GAPDH mRNA expression and represent means \pm SEM ($n = 5$) of values obtained for detectable cytokines in zymosan A injected knees. Cytokine expression in saline injected control knees was low or undetectable in all animals.

observations in AIA and ZIA further suggest a greater sensitivity to agents stimulating the innate immune responses in leptin or leptin signalling deficient animals, as opposed to attenuated inflammation in models involving T-cell responses, in particular Th1-mediated diseases [4]. Indeed, both T and B lymphocytes participate in the mechanisms that lead to articular inflammation in AIA, whereas ZIA exclusively involves the innate immune response.

Leptin was previously reported to play an important role in T-cell-mediated immune responses. Evidence of defective cell-mediated immunity and lymphoid atrophy, analogous to those observed in chronic under-nutrition in humans, are detected in *ob/ob* and *db/db* mice [23-25]. Leptin stimulates the proliferation of CD4⁺ T cells and promotes

Th1 responses [6]. Congenital leptin deficiency in humans is associated with a decreased number of circulating CD4⁺ T cells, impaired T-cell proliferation and cytokine release, all of which could be reversed by the administration of recombinant leptin [26]. In addition, the OB-Rb receptor is also expressed on B cells and may participate in the development of humoral responses [14]. Consistent with these findings, leptin deficient mice are protected from inflammation mediated by T and B cells in different disease models, including AIA, experimental autoimmune encephalomyelitis, type 1 diabetes and experimental colitis [11,12,14,27].

Our results in ZIA suggest that chronic leptin deficiency interferes with adequate control of the inflammatory reaction. Protective effects of leptin were previously observed in studies of other experimental models conducted to explore innate immune responses. *Ob/ob* mice are significantly more susceptible to lipopolysaccharide (LPS)-induced death, and this feature can partly be reversed by administration of leptin [28]. OB receptor deficient *fa/fa* rats also exhibit enhanced LPS-induced hepatotoxicity [29]. Similarly, *ob/ob* and *db/db* mice are more likely to succumb after administration of TNF- α . The protective role of leptin against TNF- α induced toxicity was further supported by the deleterious effect of neutralizing anti-leptin antibodies administered to TNF- α injected mice [30]. The mechanisms underlying these protective effects of leptin are still unclear. Although thymic and circulating lymphocytes are reduced, a fourfold increase in the number of circulating monocytes was observed in leptin deficient mice, suggesting enhanced responses to monocyte activators [7]. Furthermore, an imbalance between proinflammatory and anti-inflammatory monokines has been observed in *ob/ob* mice injected with LPS, with plasma levels of the anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist being lower in leptin deficient than in normal mice [28]. However, LPS or TNF- α mediated systemic inflammation is a complex syndrome, and susceptibility to these systemic stimuli might also be influenced by the effects of leptin on nervous, endocrine, or other responses, independent of the production of inflammatory mediators.

Intravenous injection of *Staphylococcus aureus* results in a severe form of septic arthritis in mice, which is associated with decreased circulating levels of leptin. In this model, treatment with leptin significantly decreased the severity of septic arthritis without interfering with staphylococcal load in the joints [31]. The levels of IL-6 were significantly lower in mice with septic arthritis after administration of leptin [31]. Consistent with these findings, our results in ZIA indicate that IL-6 levels were higher in *ob/ob* mice than in controls. IL-6 plays an important role in turning acute inflammation into a chronic synovitis, as demonstrated by limited duration of ZIA in IL-6 deficient mice [32]. In addition, IL-6 has also been shown to play a major role in other

models of arthritis [33,34]. Thus, control of IL-6 production may be one of the mechanisms by which leptin is involved in the control of the inflammatory response during ZIA.

It is noteworthy that anaesthesia, skin cut and intra-articular injection with saline slightly enhanced serum levels of IL-6 and SAA in *ob/ob* and *+/?* mice, although to a lesser extent than in animals infected with zymosan A. Interestingly, this small, zymosan A independent inflammatory response was also greater in *ob/ob* mice than in lean controls, further supporting the presence of an inappropriate control of inflammatory responses in leptin deficiency.

Corticosterone secretion is known to be elevated in all forms of leptin deficiency and in leptin insensitivity [21,22]. However, despite the presence of elevated levels of glucocorticoids, *ob/ob* mice still exhibited a more pronounced acute phase response and longer lasting arthritis than did controls. Thus, it is conceivable that leptin deficiency could result in an even more severe form of arthritis in the absence of hypercorticosteronaemia.

The mRNA levels of different cytokines were determined in arthritic and control joints at two time points. Consistent with a previous report [17], the levels of IL-1 α , IL-1 β and IL-6 mRNA were increased during ZIA. In addition, we also detected elevated levels of IL-1 receptor antagonist and MIF, which to the best of our knowledge have not previously been reported in the joint during ZIA. MIF is a broad-spectrum proinflammatory cytokine that is implicated both in animal models of immune-mediated arthritis and in human rheumatoid arthritis [35]. The levels of mRNAs encoding these different cytokines, including IL-6, were not different between *ob/ob* mice and their lean controls. However, we cannot exclude variations in post-transcriptional regulation, which might still result in different levels of active proteins at the site of inflammation.

Conclusion

Our results indicate that resolution of ZIA is delayed in leptin and leptin receptor deficient animals. Like in other experimental models involving the innate immune response, leptin deficiency thus appears to cause inadequate control of the inflammatory response during ZIA, leading to increased joint swelling, acute phase response and delayed resolution of acute articular inflammation.

Competing interests

None declared.

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References

- Ahima RS, Flier JS: **Leptin**. *Annu Rev Physiol* 2000, **62**:413-437.
- Trayhurn P: **Thermoregulation in the diabetic-obese (db/db) mouse. The role of non-shivering thermogenesis in energy balance**. *Pflugers Arch* 1979, **380**:227-232.
- Mantzoros CS: **Role of leptin in reproduction**. *Ann N Y Acad Sci* 2000, **900**:174-183.
- Fantuzzi G, Faggioni R: **Leptin in the regulation of immunity, inflammation, and hematopoiesis**. *J Leukoc Biol* 2000, **68**:437-446.
- Faggioni R, Feingold KR, Grunfeld C: **Leptin regulation of the immune response and the immunodeficiency of malnutrition**. *FASEB J* 2001, **15**:2565-2571.
- Lord GM, Matarese G, Howard JK, Baker RJ, Bloom SR, Lechler RI: **Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression**. *Nature* 1998, **394**:897-901.
- Faggioni R, Jones-Carson J, Reed DA, Dinarello CA, Feingold KR, Grunfeld C, Fantuzzi G: **Leptin-deficient (ob/ob) mice are protected from T cell-mediated hepatotoxicity: role of tumor necrosis factor alpha and IL-18**. *Proc Natl Acad Sci USA* 2000, **97**:2367-2372.
- Martin-Romero C, Santos-Alvarez J, Goberna R, Sanchez-Margalet V: **Human leptin enhances activation and proliferation of human circulating T lymphocytes**. *Cell Immunol* 2000, **199**:15-24.
- Lord GM, Matarese G, Howard JK, Bloom SR, Lechler RI: **Leptin inhibits the anti-CD3-driven proliferation of peripheral blood T cells but enhances the production of proinflammatory cytokines**. *J Leukoc Biol* 2002, **72**:330-338.
- Matarese G, Sanna V, Di Giacomo A, Lord GM, Howard JK, Bloom SR, Lechler RI, Fontana S, Zappacosta S: **Leptin potentiates experimental autoimmune encephalomyelitis in SJL female mice and confers susceptibility to males**. *Eur J Immunol* 2001, **31**:1324-1332.
- Matarese G, Di Giacomo A, Sanna V, Lord GM, Howard JK, Di Tuoro A, Bloom SR, Lechler RI, Zappacosta S, Fontana S: **Requirement for leptin in the induction and progression of autoimmune encephalomyelitis**. *J Immunol* 2001, **166**:5909-5916.
- Matarese G, Sanna V, Lechler RI, Sarvetnick N, Fontana S, Zappacosta S, La Cava A: **Leptin accelerates autoimmune diabetes in female NOD mice**. *Diabetes* 2002, **51**:1356-1361.
- Sanna V, Di Giacomo A, La Cava A, Lechler RI, Fontana S, Zappacosta S, Matarese G: **Leptin surge precedes onset of autoimmune encephalomyelitis and correlates with development of pathogenic T cell responses**. *J Clin Invest* 2003, **111**:241-250.
- Busso N, So A, Chobaz-Peclat V, Morard C, Martinez-Soria E, Talabot-Ayer D, Gabay C: **Leptin signaling deficiency impairs humoral and cellular immune responses and attenuates experimental arthritis**. *J Immunol* 2002, **168**:875-882.
- Keystone EC, Schorlemmer HU, Pope C, Allison AC: **Zymosan-induced arthritis: a model of chronic proliferative arthritis following activation of the alternative pathway of complement**. *Arthritis Rheum* 1977, **20**:1396-1401.
- Takeuchi O, Akira S: **Toll-like receptors; their physiological role and signal transduction system**. *Int Immunopharmacol* 2001, **1**:625-635.
- van de Loo FA, Kuiper S, van Enckevort FH, Arntz OJ, van den Berg WB: **Interleukin-6 reduces cartilage destruction during experimental arthritis. A study in interleukin-6-deficient mice**. *Am J Pathol* 1997, **151**:177-191.
- Palmer G, Talabot-Ayer D, Szalay-Quinodoz I, Maret M, Arend WP, Gabay C: **Mice transgenic for intracellular interleukin-1 receptor antagonist type 1 are protected from collagen-induced arthritis**. *Eur J Immunol* 2003, **33**:434-440.
- Sipe JD, Gonnerman WA, Loose LD, Knapschaefer G, Xie WJ, Franzblau C: **Direct binding enzyme-linked immunosorbent assay (ELISA) for serum amyloid A (SAA)**. *J Immunol Methods* 1989, **125**:125-135.
- Walker CD, Sizonenko PC, Aubert ML: **Modulation of the neonatal pituitary and adrenocortical responses to stress by thyroid**

- hormones in the rat: effects of hypothyroidism and hyperthyroidism. *Neuroendocrinology* 1989, **50**:265-273.
21. Coleman DL, Burkart DL: **Plasma corticosterone concentrations in diabetic (db) mice.** *Diabetologia* 1977, **13**:25-26.
 22. Guillaume-Gentil C, Rohner-Jeanrenaud F, Abramo F, Bestetti GE, Rossi GL, Jeanrenaud B: **Abnormal regulation of the hypothalamo-pituitary-adrenal axis in the genetically obese fa/fa rat.** *Endocrinology* 1990, **126**:1873-1879.
 23. Chandra RK: **Cell-mediated immunity in genetically obese C57BL/6J ob/ob mice.** *Am J Clin Nutr* 1980, **33**:13-16.
 24. Fernandes G, Handwerker BS, Yunis EJ, Brown DM: **Immune response in the mutant diabetic C57BL/Ks-dt+ mouse. Discrepancies between in vitro and in vivo immunological assays.** *J Clin Invest* 1978, **61**:243-250.
 25. Howard JK, Lord GM, Matarese G, Vendetti S, Ghatei MA, Ritter MA, Lechler RI, Bloom SR: **Leptin protects mice from starvation-induced lymphoid atrophy and increases thymic cellularity in ob/ob mice.** *J Clin Invest* 1999, **104**:1051-1059.
 26. Farooqi IS, Matarese G, Lord GM, Keogh JM, Lawrence E, Agwu C, Sanna V, Jebb SA, Perna F, Fontana S, Lechler RI, DePaoli AM, O'Rahilly S: **Beneficial effects of leptin on obesity, T cell hyporesponsiveness, and neuroendocrine/metabolic dysfunction of human congenital leptin deficiency.** *J Clin Invest* 2002, **110**:1093-1103.
 27. Siegmund B, Lehr HA, Fantuzzi G: **Leptin: A pivotal mediator of intestinal inflammation in mice.** *Gastroenterology* 2002, **122**:2011-2025.
 28. Faggioni R, Fantuzzi G, Gabay C, Moser A, Dinarello CA, Feingold KR, Grunfeld C: **Leptin deficiency enhances sensitivity to endotoxin-induced lethality.** *Am J Physiol* 1999, **276**:R136-142.
 29. Yang SQ, Lin HZ, Lane MD, Clemens M, Diehl AM: **Obesity increases sensitivity to endotoxin liver injury: implications for the pathogenesis of steatohepatitis.** *Proc Natl Acad Sci USA* 1997, **94**:2557-2562.
 30. Takahashi N, Waelput W, Guisez Y: **Leptin is an endogenous protective protein against the toxicity exerted by tumor necrosis factor.** *J Exp Med* 1999, **189**:207-212.
 31. Hultgren OH, Tarkowski A: **Leptin in septic arthritis: decreased levels during infection and amelioration of disease activity upon its administration.** *Arthritis Res* 2001, **3**:389-394.
 32. de Hooge AS, van De Loo FA, Arntz OJ, van Den Berg WB: **Involvement of IL-6, apart from its role in immunity, in mediating a chronic response during experimental arthritis.** *Am J Pathol* 2000, **157**:2081-2091.
 33. Ohshima S, Saeki Y, Mima T, Sasai M, Nishioka K, Nomura S, Kopf M, Katada Y, Tanaka T, Suemura M, Kishimoto T: **Interleukin 6 plays a key role in the development of antigen-induced arthritis.** *Proc Natl Acad Sci USA* 1998, **95**:8222-8226.
 34. Alonzi T, Fattori E, Lazzaro D, Costa P, Probert L, Kollias G, De Benedetti F, Poli V, Ciliberto G: **Interleukin 6 is required for the development of collagen-induced arthritis.** *J Exp Med* 1998, **187**:461-468.
 35. Morand EF, Leech M, Weedon H, Metz C, Bucala R, Smith MD: **Macrophage migration inhibitory factor in rheumatoid arthritis: clinical correlations.** *Rheumatology (Oxford)* 2002, **41**:558-562.