Research Article

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A bidirectional crosstalk between iNKT cells and adipocytes mediated by leptin modulates susceptibility for T cell mediated hepatitis

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Background & Aims: Immunometabolism is an emerging field of clinical investigation due to the obesity epidemic worldwide. A reciprocal involvement of immune mediators in the body energy metabolism has been recognized for years, but is only partially understood. We hypothesized that the adipokine leptin could provide an important modulator of iNKT cells.

Methods: The expression of leptin receptor (LR) on resting and activated iNKT cells was measured by flow cytometry. FACS-sorted hepatic iNKT cells were stimulated with anti-CD3/CD28Ab coated beads in the absence or presence of a neutralizing anti-leptin Ab. Furthermore, we evaluated the outcome of LR blocking nanobody treatment in ConA induced hepatitis and towards metabolic parameters in WT and iNKT cell deficient mice.

Results: The LR is expressed on iNKT cells and leptin suppresses iNKT cell proliferation and cytokine production *in vitro*. LR deficient iNKT cells are hyper-responsive further enforcing the role of leptin as an important inhibitor of iNKT cell function. Consistently, *in vivo* blockade of LR signaling exacerbated ConA hepatitis in wild-type but not in iNKT cell deficient mice, through both Janus kinase (JAK)2 and mitogen-activated protein kinase (MAPK) dependent mechanisms. Moreover, LR inhibition altered fat pad features and was accompanied by insulin resistance, only in wild-type mice. Curiously, this interaction was strictly dependent on MAPK mediated LR signaling in iNKT cells and uncoupled from the more central effects of leptin.

Conclusions: Our data support a new concept of immune regulation by which leptin protects towards T cell mediated hepatitis via modulation of iNKT cells.

Keywords: Immunology; Liver pathology; Immunometabolism; Adipokines; ConA.

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Abbreviations: α-GalCer, α-galactosylceramide; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ConA, concanavalin A; iNKT cell, invariant natural killer T cell; LR, leptin receptor; MAPK, mitogen-activated protein kinase; mAlb, mouse serum albumin specific nanobody; n.d., not detectable; SOCS, suppressor of cytokine signaling; WAT, white adipose tissue.

Introduction

Invariant natural killer T (iNKT) cells constitute an immunoregulatory and conserved T cell sublineage with several unique characteristics. The most striking feature is the limited T cell receptor (TCR) diversity that comprises an invariant α-chain (Vα14-Jα18 in mice) combined with a restricted set of β-chains [1]. Unlike conventional T cells which recognize peptide antigens, iNKT cells recognize glycolipid antigens that include microbially derived or self antigens, presented by the MHC class I like molecule CD1d. The exogenous glycolipid α-galactosylceramide (α-GalCer) has been recognized for a long time as a strong agonist of iNKT cells. Upon stimulation, iNKT cells massively proliferate and rapidly secrete a broad scale of cytokines, both pro- and anti-inflammatory [2]. Consequently, they profoundly modulate immune responses in health and a variety of diseases [2].

It has been established for many years that immune cells can be influenced by adipokines, cytokines and hormones produced by white adipose tissue (WAT). More recently, new evidence points to a reciprocal involvement of immune mediators in the body energy metabolism, which has led to the new field of immunometabolism [3]. Interestingly, iNKT cells are abundantly

present in adipose tissues both in mice and humans [4–6], and several studies suggest a role for iNKT cells, although still controversial, in obesity-induced inflammation and metabolic functions [5–10]. Moreover, recent reports show that adipocytes can activate iNKT cells directly in a CD1d dependent manner [5,11], but the crosstalk between these cells remains poorly understood. Adipokines such as leptin might influence the function of iNKT cells. Leptin is a class I cytokine that "reports" the nutritional and energy status of the body to the brain. Circulating levels are proportional to the body fat mass and, through its receptor in the hypothalamus, leptin acts as an anorexigenic factor [12]. Furthermore, leptin is a pleiotropic molecule that is crucial for proper immune responses as reflected in both leptin (*ob*/*ob*) and leptin receptor (*db*/*db*) deficient mice, who, next to clear signs of obesity, show profound immune aberrations [13,14]. As such, leptin allows input on the body's nutritional status in the regulatory network of cytokines that controls immune function. We hypothesized that leptin could provide an important molecular link between adipose tissue and glycolipid reactive T cells in the adult immune system.

Materials and methods

Animals and housing

Jα18$^{+/+}$, *Jα18*$^{-/-}$ [15] and Lck-*SOCS3* Tg mice [16], all on the C57BL/6 background, were kept and bred in pathogen–free conditions in accordance with the general recommendations for animal breeding and housing. Obese *db*/*db* and lean littermate control mice were obtained from The Jackson Laboratory. Sex and age (between 8 and 12 wk) matched animals were used in each experiment. Experiments were conducted according to the guidelines of the Ethics Committee of Laboratory Animals Welfare of Ghent University.

Reagents

Antibodies were purchased from eBioscience Europe (Vienna, Austria) unless stated otherwise. α-GalCer was synthesized at the Department of Medicinal Chemistry (Ghent University, Belgium). Bispecific 2.17-mAlb nanobodies were constructed as described before [17].

Lymphocyte and iNKT isolation and culture

Hepatic lymphocyte and splenocytes were isolated as described previously [18] and were further used for flow cytometry, *in vitro* cell cultures or iNKT cell purification. Splenic lymphocytes (6×10^5) were cultured in presence of α-GalCer (200 ng/ml) and the supernatant was collected after 72 or 96 h for cytokine quantification. For *in vitro* iNKT assays, hepatic iNKT cells were sorted with a FACS Aria II cell-sorter (BD), based on the expression of CD5 and NK1.1. This combination of markers was applied to guarantee that cells were not activated prior to the culture set-up [19]. A representative pre and post-sort profile of the cells is shown in Supplementary Fig. 1. CD5$^+$NK1.1^{int} iNKT cells (>94% α-GalCer-CD1d tetramer positive) were cultured in 96-well plates (10^5) and stimulated with anti-mouse CD3/CD28 Dynabeads (Dynal) or IL-12 (1 ng/ml) combined with IL-18 (10 ng/ml) with or without an anti-mouse leptin neutralizing antibody (R&D Systems) for 3 days, and proliferation tested by a [^{3}H]-thymidine incorporation assay. In parallel, iNKT cells were stimulated *in vitro* with anti-CD3/CD28 coated beads in the presence of absence of 1 μg/ml recombinant leptin (R&D) in serum-free X-VIVO medium (Lonza, Belgium). Cytokine production was determined using Cytokine Bead Arrays (CBA, BD Biosciences).

Flow cytometry

LR expression on hepatic and splenic lymphocytes was assessed using anti-mouse LR antibodies (clone 4A9 and 1G2). Lymphocytes were additionally stained with 7-AAD (BD), anti-mouse TCR-β antibody (eBioscience) and α-GalCer-loaded CD1d tetramers to gate on viable T cells (7-AAD$^-$TCRβ$^+$Tetramer$^-$) and iNKT cells (7-AAD$^-$TCRβ$^+$Tetramer$^+$) as described before [18]. Samples were measured on a FACS Canto II (BD Biosciences) and analyzed using FlowJo analysis software (Tree Star Inc., Ashland, Oregon, USA). Representative data plots of the T/iNKT cell gating strategy are shown in Supplementary Fig. 2.

ConA induced hepatitis

Hepatitis was induced in mice by intravenous injection of concanavalin A (ConA, 20 mg/kg; Sigma-Aldrich, Bornem, Belgium). Blood was collected before and 8 and 24 h after ConA challenge for determination of serum ALT and AST levels (by photometric analysis). After 24 h mice were sacrificed and livers were dissected and prepared for histological examination. For adoptive transfer experiments, hepatic iNKT cells were isolated with PE-labeled α-GalCer-loaded CD1d tetramers and anti-PE microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) by magnetic purification and injected in spleens of Jα18 knockout mice according to [20].

LRb mRNA detection by RT-PCR

RNA isolation from sorted hepatic CD5$^+$ NK1.1^{int} iNKT cells and subsequent first-strand cDNA synthesis was performed using the μMACS™ One-step cDNA Synthesis Kit (Miltenyi Biotec). LRb and actin PCR amplification details are described in Supplementary Materials and methods.

Leptin receptor signaling blockade in vivo

Mice received a daily i.p. injection of 2.17-mAlb (day1–3: 300 μg/mouse/day and day 4–7: 200 μg/mouse/day) or PBS during one week (between 8 and 9 AM). For indicated experiments, mice were additionally i.p. injected with chemical inhibitors AG490 and PD98059 (both from LC Laboratories, Woburn, MA, USA) or DMSO (Sigma-Aldrich, Bornem, Belgium) for 8 consecutive days starting one day before the first 2.17-mAlb injection. Inhibitors were dissolved in DMSO at 2.5 mg/ml and mice received 5 mg/kg/day. Activity and specificity of inhibitors was verified *in vitro* (Supplementary Fig. 4).

Metabolic parameters

The body weight of each mouse was daily monitored between 8 and 9 AM. The delta weight increase as compared to the initial weight was calculated for each repeated day (Δweight dx-d1). At the end of the 2.17-mAlb/PBS treatment, blood samples of mice were collected and glucose levels were measured (OneTouchVita™, LifeScan). Insulin (by ELISA, Mercodia), tryglycerides and cholesterol levels (both by spectrophotometry on a Cobas 8000 c701; Roche Diagnostics) were measured in corresponding serum samples. Livers and perigonadal fat pads were dissected, weighted and prepared for histological examination.

Histology

Liver and perigonadal fat pads were fixed in 4% formaldehyde, processed for paraffin-embedded sections and stained with hematoxylin and eosin (H&E) according to standards protocols. Details of histological analyses are described in Supplementary Materials and methods.

Statistics

Results are expressed as mean ± SEM unless indicated otherwise in Figure legends using Prism software v5.0 (GraphPad, San Diego, CA, USA). Differences between two data groups were assessed by Mann-Whitney U tests or Student's *t* tests, performed using SPSS 15 statistical software (Chicago, Illinois, USA). $^*p < 0.05$ and $^{**}p < 0.01$.

Results

Leptin receptor expression on resting and activated iNKT cells

The LR is abundantly expressed on a myriad of immune cells, including monocytes, macrophages, T cells and NK cells but its