

Interleukin-6 autoantibodies are involved in the pathogenesis of a subset of type 2 diabetes

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Abstract

Interleukin-6 (IL6) is critically involved in inflammation and metabolism. About 1% of people produce IL6 autoantibodies (aAb-IL6) that impair IL6 signaling *in vivo*. We tested the hypothesis that the prevalence of such aAb-IL6 is increased in type 2 diabetic patients and that aAb-IL6 plays a direct role in causing hyperglycemia. In humans, the prevalence of circulating high-affinity neutralizing aAb-IL6 was 2.5% in the type 2 diabetic patients and 1% in the controls (odds ratio 2.5, 95% confidence interval 1.2–4.9, $P=0.01$). To test for the role of aAb-IL6 in causing hyperglycemia, such aAb-IL6 were induced in mice by a validated vaccination procedure. Mice with plasma levels of aAb-IL6 similar to the 2.5% type 2 diabetic patients developed obesity and impaired glucose tolerance (area under the curve (AUC) glucose,

2056 ± 62 vs 1793 ± 62 , $P=0.05$) as compared with sham-vaccinated mice, when challenged with a high-fat diet. Mice with very high plasma levels of aAb-IL6 developed elevated fasting plasma glucose (mM, 4.8 ± 0.4 vs 3.3 ± 0.1 , $P<0.001$) and impaired glucose tolerance (AUC glucose, 1340 ± 38 vs 916 ± 25 , $P<0.001$) as compared with sham-control mice on normal chow. In conclusion, the prevalence of plasma aAb-IL6 at levels known to impair IL6 signaling *in vivo* is increased 2.5-fold in people with type 2 diabetes. In mice, matching levels of aAb-IL6 cause obesity and hyperglycemia. These data suggest that a small subset of type 2 diabetes may in part evolve from an autoimmune attack against IL6.

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Introduction

A complex interplay of pre- and post-natal environmental factors and polygenetics is involved in the pathogenesis of type 2 diabetes (Andersen *et al.* 2005). Prominent pathological features include insulin resistance, inability in a timely fashion to produce adequate amounts of insulin in response to rises in blood glucose, and chronic low-grade inflammation (Andersen *et al.* 2005, Cai *et al.* 2005).

Interleukin (IL)-6 appears to be a key mediator of inflammation (Nishimoto & Kishimoto 2006), albeit IL6 gene deficiency is tolerated with only minor defects in inflammation and immunity (Kopf *et al.* 1994, 1995). Physical inactivity (Fischer *et al.* 2007) is associated with chronically elevated plasma IL6 levels that have been related to the development of type 2 diabetes (Pickup *et al.* 1997). On the other hand, IL6-deficient mice develop mature-onset obesity and glucose intolerance (Wallenius *et al.* 2002). This metabolic phenotype is attributed to downstream

insufficient AMPK activity (Kelly *et al.* 2004) and reduced exercise capacity (Falldt *et al.* 2004). Also, a pivotal role for hepatic IL6 signaling in insulin-induced inhibition of hepatic gluconeogenesis may contribute to the impaired glucose tolerance seen in IL6-deficient mice (Inoue *et al.* 2006). Altogether, while chronically elevated IL6 levels may exert detrimental effects on insulin signaling in hepatocytes and myocytes, a minimum/normal IL6 activity seems necessary to maintain normal metabolic control.

IL6 is secreted into the extracellular space and signals via the cognate IL6 receptor (IL6R or IL6RA as listed in the MGI Database) on the surface of primary target cells. The IL6R is juxta-positioned to the glycoprotein 130 (GP130) signal transduction unit and together they form the cellular IL6R system (Taga & Kishimoto 1997). Both the IL6R and the GP130 are also shed from the plasma membranes and circulate in soluble forms. Binding of IL6 to the soluble IL6R renders cells sensitive to IL6/IL6R trans-signaling via direct interaction with the globally expressed GP130 receptor

subunit, and soluble GP130 inhibits IL6 trans-signaling (Kallen 2002, Richards *et al.* 2006, Rose-John *et al.* 2006).

Substantial high-affinity binding of IL6 is detectable in highly diluted plasma from around 1% of apparently healthy blood donors (Galle *et al.* 2004b). This binding is not related to soluble IL6R compounds but evidently due to nanomolar concentrations of *in vitro* neutralizing polyclonal IgG autoantibodies (aAb) with very high binding affinity (Hansen *et al.* 1991, 1993, Galle *et al.* 2004b). In fact, most of these aAb-IL6 are IgG1 antibodies (Hansen *et al.* 1993, 1995). Like almost all known aAb, there is no explanation for their induction, and their presence among apparently healthy individuals, such as blood donors, indicates that they are tolerated by many people, at least for some time, without causing overt pathology. There is circumstantial evidence that individuals having high circulating levels of aAb-IL6 are somewhat IL6 deficient (Hansen *et al.* 1993, 1995, 2007, Galle *et al.* 2004a,b).

Here, we demonstrate that plasma levels of aAb-IL6 that prevent IL6 function *in vivo* are more prevalent in people with type 2 diabetes compared with control persons. In parallel, we addressed a possible causative role of aAb-IL6 in obesity and/or type 2 diabetes by inducing different levels of circulating aAb-IL6 in mice. Mice having aAb-IL6 levels pertinent to the human situation display several metabolic features of type 2 diabetes including fasting hyperglycemia and glucose intolerance, obesity, and dyslipidemia, depending on the circulating level of aAb-IL6 and the diet. Collectively, these data suggest that aAb-IL6 may be involved in the pathogenesis of a subset of type 2 diabetes.

Materials and Methods

Human study populations

Plasma levels of aAb-IL6 were measured in four study groups: A) a group of 569 unrelated type 2 diabetic patients (men/women, 336/233; mean \pm s.d. age, 60.5 \pm 11 years; body mass index (BMI), 29.1 \pm 5.3 kg/m²) recruited at the outpatient clinic of Steno Diabetes Centre, Copenhagen; B) a group of 233 unrelated persons (men/women, 115/118; mean \pm s.d. age, 51.7 \pm 14 years; BMI, 25.3 \pm 3.8 kg/m²) with normal glucose tolerance recruited randomly from Copenhagen County; C) a group of 563 randomly selected blood donors with available information on age and sex (men/women/unknown, 289/176/98; mean \pm s.d. age, 38.3 \pm 11 years) from the Blood Bank of Rigshospitalet, Copenhagen, and D) a group of 2000 randomly selected blood donors from the Blood Bank of Rigshospitalet, Copenhagen. Informed written consent was obtained from all study participants. All the study participants were unrelated Danish whites by self-report. The studies were conducted in accordance with the Declaration of Helsinki II and approved by the local Ethical Committee of Copenhagen and Frederiksberg.

RIA for aAb-IL6 in humans and mice

The aAb-IL6 were measured by a validated RIA as previously described (Hansen *et al.* 1995, 2007, Galle *et al.* 2004a,b). In brief, recombinant IL6 was labeled with ¹²⁵I using the chloramine-T method and purified as monomeric ¹²⁵I-IL6 by means of molecular size chromatography on Sephadex G-75 superfine (Amersham Biosciences) to yield tracers with specific activities varying between 150 and 200 c.p.m./pg. More than 95% of the tracers could be bound specifically and in a saturable manner to polyclonal anti-IL6 antibodies, U-937 cells (human IL6), and B9 cells (murine IL6; Hansen *et al.* 1995). Plasma (25%) was mixed with 3000 c.p.m. ¹²⁵I-IL6 in a final volume of 40 μ l PBS buffer. After incubation at 4 °C for 18 h, immune complexes were separated at 4 °C by affinity chromatography on protein-G Sepharose Fast flow (Amersham Biosciences) after which IgG-bound and free ¹²⁵I-IL6 were counted. In this particular set up, we previously assessed a significant *in vivo* IL6 inhibition by aAb-IL6 when 40% (mean \pm s.d., 39.5 \pm 4.8%) or more of the total ¹²⁵I-IL6 were bound to picomolar affinity IgG (bound/total (B/T)) > 0.40; Galle *et al.* 2004a,b, Hansen *et al.* 2007). A sample was considered positive for aAb-IL6 if more than 3% of the tracer was IgG bound and could be displaced with a surplus of unlabeled rIL6. All measurements were done in duplicates with intra-assay and inter-assay coefficient of variations below 0.08. A B/T value of 0 designates a level of aAb-IL6 below the detection limit, and a B/T level of 0.40 was used as an operational antibody activity where *in vivo* neutralization has been validated and where 70–99.9% of 10 pM of circulating IL6 are bound to aAb-IL6 (Galle *et al.* 2004a,b, Hansen *et al.* 2007).

Animal studies

Vaccination induced aAb-IL6 Wild-type murine IL6 and eight highly immunogenic IL6 analogs were expressed in *Escherichia coli* with an N-terminal 6 \times His-tag as previously reported (Galle *et al.* 2004a). The eight analogs were pooled and adsorbed to Al(OH)₃ (Alhydrogel 1.3% Brenntag Biosector AIS, Frederikssund, Denmark) and administered s.c. in the neck of mice at 3-week intervals as follows: 100 μ g/ml \times 3 times (Protocol A) or 10 μ g/ml \times 4 times (Protocol B). Control mice were sham vaccinated with Al(OH)₃ alone (sham-control).

Animals Experiments were initiated in male DBA/1 mice (Taconic M&B, Ejby, Denmark) aged 5–6 weeks. Animals were kept in a temperature-controlled environment (22–23 °C) at a 12 h light:12 h darkness cycle (lights on at 0600 am) with free access to food and water unless otherwise stated. All the experiments were conducted in accordance with internationally accepted guidelines for the care and use of laboratory animals and approved by the Danish Committee for Animal Research.

Experiment A After 1 week of acclimatization, the mice were divided into two groups and vaccinated with IL6 analogs or sham vaccine according to Protocol A. IL6 analog-vaccinated mice developed aAb-IL6 with $B/T > 0.95$ (termed aAb-IL6-KO), whereas sham-vaccinated mice did not develop detectable levels of aAb-IL6 (sham-control). Four weeks following the last injection, a blood sample was obtained and the mice were subjected to oral glucose tolerance tests as detailed below.

Experiment B Animals were treated as described above and vaccinated according to Protocol B. IL6 analog-vaccinated mice developed levels of aAb-IL6 with $0.40 < B/T < 0.80$ (pertinent to the human situation, termed aAb-IL6-positive) and sham-vaccinated (sham-control) mice did not develop detectable levels of aAb-IL6. Immediately following the last vaccination, the mice were sub-divided into four groups of diet manipulation. Diets (Research Diets, New Brunswick, NJ, USA): control chow diet (D12450B, chow); high-fat diet (D12492, HFD). Regimens: 1) sham-control on chow ($n = 8$); 2) sham-control on HFD ($n = 9$); 3) aAb-IL6-positive on chow ($n = 11$); 4) aAb-IL6-positive on HFD ($n = 7$). Animals were kept on these regimens until terminated. Following 16 weeks on the diets, a blood sample was obtained and subsequently the mice were subjected to an oral glucose tolerance test as detailed below. Following an additional 6 weeks, the animals were terminated as follows: the mice were semi-fasted (given 50% of normal daily food intake the night before), and then in the morning the animals were challenged by an s.c. injection (1 mU/kg) of insulin (Actrapid, Novo Nordisk, Copenhagen, Denmark). Three hours later, the mice were briefly anesthetized with CO₂ and killed by cervical dislocation. The liver was quickly excised and freeze-clamped in liquid nitrogen and subsequently kept at -80°C for later analysis.

Experiment C Animals were subjected to the vaccination Protocol B, and subsequently all the animals were placed on an HFD (as above) for a period of 48 weeks. The phenotype that developed in experiment C was similar to that reported for experiment B (data not shown). Then the animals were subjected to an acute insulin or vehicle challenge. Regimens: 1) Sham-control vehicle challenge ($n = 3$); 2) Sham-control insulin challenge ($n = 4$); 3) aAb-IL6-positive vehicle challenge ($n = 5$); 4) aAb-IL6-positive insulin challenge ($n = 5$). Briefly, the mice were given a single s.c. injection of insulin (1 mU/kg, Actrapid, Novo Nordisk) or vehicle control, and 15 min later the mice were briefly anesthetized with CO₂ and killed by cervical dislocation. The liver as well as soleus, extensor digitorum longus (EDL), and quadriceps muscles were quickly excised and freeze-clamped in liquid nitrogen and subsequently kept at -80°C for later analysis of insulin signaling pathways.

Oral glucose tolerance test An oral glucose tolerance test was performed in mice as previously described (Vrang *et al.* 2006). Briefly, tail-blood glucose was

measured before ($t = 0$ min) and after ($t = 15, 30, 45, 60, 120,$ and 180 min) an oral glucose load of 2 g/kg was given to the mice at $t = 0$ min, and the glucose tolerance was evaluated by calculation of the area under the glucose disappearance curve.

Blood chemistry Blood sample (200 μl) from the orbital plexus of 17-h fasted mice was collected in ice-cooled EDTA tubes. After centrifugation, plasma was kept at -80°C for later analysis. The plasma concentrations of triacylglycerol and total cholesterol were determined on a Hitachi 912 autoanalyzer (Boehringer Mannheim). Plasma levels of insulin and leptin were determined by a Linco kit (LincoResearch, St Charles, MS, USA) using a Luminex analyzer (Luminex Corporation, Austin, TX, USA).

Real-time PCR Fifteen milligrams of liver tissue were used for RNA extraction using the TRIzol method (Invitrogen) as previously described (Keller *et al.* 2004). Two micrograms of the extracted RNA were reversely transcribed (Applied Biosystems Taqman RT-Kit, Foster City, CA, USA). The cDNA was prepared using random hexamers. The mRNA content of a given gene was determined by PCR using real-time PCR with Taqman probes (ABIPRISM 7900 Sequence Detection System, Applied Biosystems). Commercially available primers and probes (Applied Biosystems) were used for identification of 18S, fibrinogen, haptoglobin, phosphoenolpyruvate carboxykinase-1 (PEPCK or PCK1 as listed in the MGI Database), glucose 6-phosphatase (Glu-6-Pase or G6PC as listed in the MGI Database; Applied Biosystems numbers: Mm00513575_m1, Mm00516884_m1, Mm00440636_m1, Mm00839363_m1 respectively). The mRNA levels were determined from the individual threshold cycle and the standard curve method. As the 18S-rRNA level was similar between groups of animals, it was suitable as an endogenous control in the present study. Thus, the level of mRNA for each target gene was adjusted to the level of 18S-rRNA in the same tissue sample.

Western blot

STAT-3. Livers were homogenized at 4°C in modified RIPA cell lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.25% Na deoxycholate, 1% Triton X-100, 1 $\mu\text{g/ml}$ pepstatin A, 1 mM Na₃VO₄, 1 mM NaF, and protease inhibitor cocktail tablets (Roche Diagnostics)). The lysates were rotated for 1 h at 4°C and then centrifuged (13 000 g for 1 h). Total protein content was determined using the Bradford method (Bio-Rad). Equal amounts of liver protein were separated by SDS-PAGE, and subsequently transferred to polyvinylidene fluoride membranes (Amersham Pharmacia Biotech). Membranes were incubated overnight with anti-phospho-STAT3 (Tyr705; Cell Signaling Technology, product number 9145, Danvers, MA, USA) and anti-STAT3 (Santa Cruz Biotechnology, product number L2905, Santa Cruz, CA, USA). The appropriate

peroxidase-conjugated secondary antibodies were from DAKO (Glostrup, Denmark). All the antibodies were diluted in 5% TOP-Blok (Biochemika, Sigma-Aldrich); the primary antibodies were diluted 1:1000 and the secondary antibodies 1:50 000. Blots were stripped and re-probed to normalize to equal protein amount. Proteins were visualized by Super Signal West Femto (Pierce, Rockford, IL, USA) and quantified using Molecular Analyst software (Bio-Rad). Results were adjusted to the sham-vaccinated mice on normal diet and reported in arbitrary units (au).

AKT, IRS1, and IR. Protein lysates from liver and muscle (EDL, soleus, and quadriceps) were prepared, and total protein content was determined as described above. Then samples of 25 µg were loaded on a Multi Spot 96-well plate (Meso Scale Discovery, Gaithersburg, MD, USA), coated with AKT, IRS1, or IR. Hereafter pAKT, total AKT, pIRS1, total IRS1, pIR, and total IR were determined by electrochemiluminescence singleplex system (Sector Imager 2400; Meso Scale Discovery) according to the instructions of the manufacturer.

Statistical analysis

Studies in humans A non-parametric two-sided Wilcoxon rank sum test was used to compare plasma levels of aAb-IL6 between cases and control persons, while a two-sided Fisher's exact test was used to test differences in the frequency of exposure. Following the assumption that the plasma binding activity of aAb-IL6 affects disease status, we used logistic regression to evaluate the impact of age and the plasma levels of aAb-IL6 on disease status. We explored three models. Model 1: a model involving age, plasma binding activity of aAb-IL6, and their linear interaction; model 2: a model involving additive effect between age and plasma binding activity of aAb-IL6 only; model 3: a model involving age only. For interaction between age and plasma levels of aAb-IL6, we compared model 1 with model 2, and for the overall effect of plasma levels of aAb-IL6 on disease status, we compared model 1 with model 3. All calculations were performed using R v.2.2.4 (www.r-project.org).

Studies in mice Data are expressed as mean ± S.E.M. and compared by an unpaired Student's *t*-test, unless otherwise stated. $P < 0.05$ was considered significant. All the statistical calculations were performed using GraphPad Prism version 4.00 (GraphPad Software, Inc., San Diego, CA, USA).

Results

Studies in humans

The aAb-IL6 were measurable in the plasma from 10% (61 of 569) of the type 2 diabetic patients and in 10% (80 of 796) of the control persons by a validated RIA as previously described (Hansen *et al.* 1995, 2007, Galle *et al.* 2004a,b).

In this particular set up, we previously assessed a significant *in vivo* IL6 inhibition by aAb-IL6, when 40% (mean ± S.D., $39.5 \pm 4.8\%$) or more of the total ^{125}I -IL6 were bound to picomolar affinity IgG ($B/T > 0.40$; Galle *et al.* 2004a,b, Hansen *et al.* 2007). Moreover, the prevalence of plasma aAb-IL6 that impair IL6 signaling *in vivo* corresponding to a maximal binding capacity above 30 pM was 2.5% among the type 2 diabetic patients and 1% in the control persons (Fig. 1 and Table 1, odds ratio 2.5 with confidence interval of 1.2–4.9, $P = 0.01$). By logistic regression of disease status, we observed a significant interaction between age and plasma aAb-IL6 binding activity (Fig. 1, model 1 versus model 2, $P = 0.04$). Further, plasma aAb-IL6 binding activity was significantly associated with disease status (model 1 versus model 3, $P = 0.002$) when considering both the main effect and the interactive effect with age.

Studies in mice

In order to elucidate whether aAb-IL6 observed in plasma of type 2 diabetic patients were diabetogenic, we examined the metabolic consequences of different circulating levels of aAb-IL6 in mice. In experiment A, a potent vaccine, was used to induce 'knockout' levels ($B/T > 0.95$) of aAb-IL6, while in experiment B we induced levels of aAb-IL6 ($0.40 < B/T < 0.80$) corresponding to the situation observed in 2.5% of type 2 diabetic patients. Furthermore, the influence of environmental factors, i.e. the composition of the diet was studied.

Mice vaccinated to induce 'knockout' levels of aAb-IL6 displayed fasting hyperglycemia and impaired glucose tolerance compared with sham-vaccinated (sham-control)

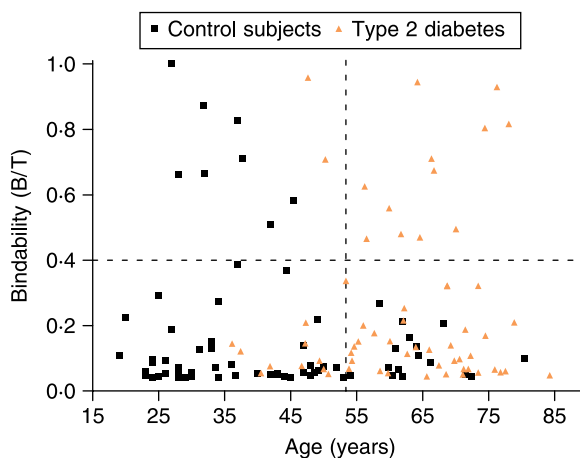


Figure 1 The prevalence of plasma aAb-IL6 that impair IL6 signaling *in vivo* was 2.5% among the type 2 diabetic patients and 1% in the control persons. There was a relationship between age and plasma binding activity of aAb-IL6 in type 2 diabetic patients. Inserted lines indicate mean age (53.4 years) and plasma cut-off level of aAb-IL6 = 0.40 ($B/T = 0.40$). The plasma aAb-IL6 binding activity showed a strong overall association with type 2 diabetes when interactions between age and plasma binding activity was included in the analysis ($P = 0.002$).

Table 1 Study persons and their plasma levels of interleukin-6 (IL6) autoantibodies

Study group	n	B/T=0	B/T>0	B/T>0.40
Type 2 diabetes (A)	569	508 (89.3)	61 (10.7)	14 (2.5)*
Control persons (B)	233	208 (89.3)	25 (10.7)	3 (1.3)
Control persons (C)	563	508 (90.2)	55 (9.8)	5 (0.9)
Control persons (D)	2000	ND	ND	20 (1.0)

IL6 autoantibodies (aAb-IL6) were measured in four study groups. (A) A group of 569 unrelated patients with type 2 diabetes (men/women, 336/233; mean \pm s.d. age, 60.5 ± 11 years; BMI, 29.1 ± 5.3 kg/m²). (B) A group of 233 unrelated persons (men/women, 115/118; mean \pm s.d. age, 51.7 ± 14 years; BMI, 25.3 ± 3.8 kg/m²) with normal glucose tolerance. (C) A group of 563 blood donors with available information on age and sex (men/women/unknown, 289/176/98; mean \pm s.d. age, 38.3 ± 11.24 years), and (D) a group of 2000 anonymous blood donors. Data are number of persons in each group (% of each group), ND, not determined. A B/T (bound/total) level = 0 equals a level of aAb-IL6 below the detection limit, and a B/T level of 0.40 is used as a functional significant antibody cut-off level where IL6 *in vivo* neutralization has been validated, see Materials and Methods for further details. * $P < 0.05$ by Fisher's exact test: type 2 diabetes (A) versus non-diabetic (B+C+D).

mice. Fasting blood glucose levels were increased (Fig. 2C, mM, 4.8 ± 0.4 vs 3.3 ± 0.1 , aAb-IL6-KO versus sham-control, $P < 0.001$), and oral glucose tolerance was impaired (Fig. 2C and D, AUC glucose, 1340 ± 38 vs 916 ± 25 , aAb-IL6-KO versus sham-control, $P < 0.001$). No differences were found in body weight (Fig. 2A) or fasting plasma cholesterol (mM, 2.8 ± 0.1 vs 2.8 ± 0.1 , aAb-IL6-KO versus sham-control, $P = \text{NS}$), whereas fasting plasma triacylglycerol levels were elevated by ~ 1.6 -fold (Fig. 2B, mM, 1.9 ± 0.12 vs 1.2 ± 0.03 , aAb-IL6-KO versus sham-control, $P < 0.001$).

Mice vaccinated to induce levels of aAb-IL6 comparable with 2.5% of the type 2 diabetes patients displayed no differences in body weight or glycemic regulation on normal mouse chow (Fig. 3A–C). However, on a HFD these mice developed a more severe obesity (Fig. 3A) and exhibited a decreased glucose tolerance compared with sham-control mice (Fig. 3B and C, AUC glucose, 2056 ± 62 vs 1793 ± 62 , aAb-IL6-positive versus sham-control, $P = 0.05$). Additionally, fasting plasma levels of leptin and insulin were elevated

in aAb-IL6-positive mice (Table 2). To validate this, mice with levels of aAb-IL6 similar to the human situation were IL6 deficient we measured the expression of the two IL6-dependent hepatic acute phase proteins haptoglobin and fibrinogen in liver tissue. The mRNA levels were significantly decreased in aAb-IL6-positive mice compared with sham-control mice (Table 3). To elucidate whether the impaired glucose tolerance observed in aAb-IL6-positive mice was related to hepatic insulin resistance, we assessed the ability of insulin to regulate key enzymes in the liver gluconeogenic pathway. On a HFD, the aAb-IL6-positive mice displayed elevated mRNA levels of *Pepck* (*Pck1*) and *Glu-6-Pase* (*G6pc*) in liver tissue samples compared with sham-control mice 3 h following a s.c. insulin challenge (Table 3, au, 1.3 ± 0.2 vs 1.1 ± 0.1 , $P = 0.13$; 1.3 ± 0.4 vs 0.5 ± 0.1 , $P = 0.05$). In accordance, a substantially decreased phosphorylation of hepatic STAT3 proteins was demonstrated in aAb-IL6-positive mice compared with sham-control mice (Table 3). We did not find any effects of aAb-IL6 on insulin-induced phosphorylation of AKT, IRS1, or IR in the liver or in soleus, gastrocnemius, or EDL muscle (experiment C, data not shown).

Discussion

It is a subject of considerable controversy how IL6 influences metabolism (Rosen & Spiegelman 2006). Here we provide novel evidence that a normal IL6 action is required to maintain glucose tolerance. In humans, we demonstrate that the prevalence of plasma levels of aAb-IL6 that impair IL6 signaling *in vivo* (Galle *et al.* 2004a) is 2.5-fold higher in type 2 diabetic patients compared with healthy control subjects (Table 1 and Fig. 1). In mice, by inducing circulating levels of aAb-IL6 pertinent to the human situation, we demonstrate that depending upon the plasma activity of aAb-IL6, age, and diet composition these antibodies induce obesity, dyslipidemia, and impaired glucose regulation (Figs 2 and 3, Tables 2 and 3).

Loss of immunological tolerance toward endogenous cellular substances and secreted proteins is involved in much

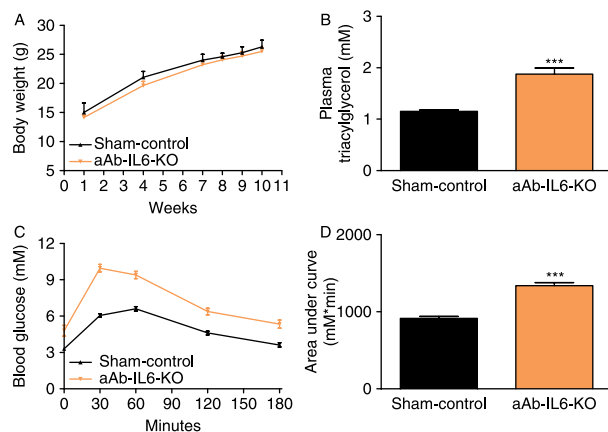


Figure 2 (A) Body weight, (B) plasma triacylglycerol, and (C and D) measures of blood glucose. Mice were IL6 vaccinated to induce high levels of IL6 autoantibodies (aAb-IL6-KO, $n = 15$, $B/T > 0.95$) or sham vaccinated ($n = 15$, aAb-IL6 $B/T = 0$, sham-control). Data are shown as average \pm s.e.m. and compared by a paired Student's *t*-test, *** $P < 0.001$.

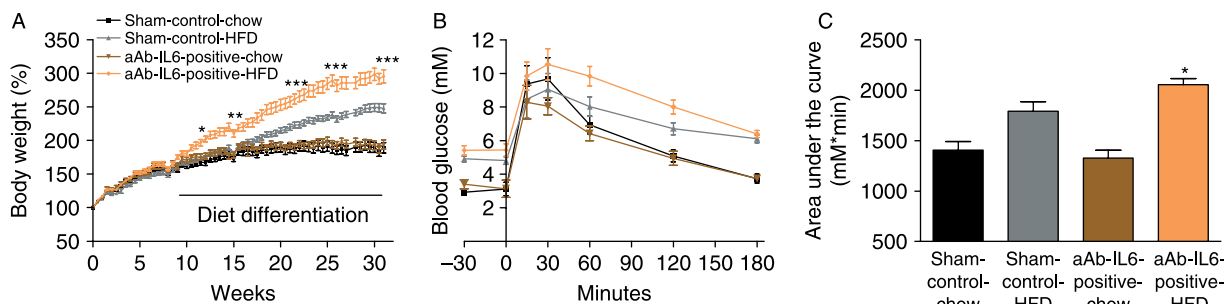


Figure 3 (A) Body weight and (B and C) measures of glucose tolerance. Mice were IL6 vaccinated to induce low plasma levels of IL6 autoantibodies (aAb-IL6-positive: $0.40 < B/T < 0.80$) or sham vaccinated (aAb-IL6: $B/T = 0$, sham-control) and exposed to a normal chow or high-fat diet (HFD). Statistics: body weight was compared by a 2-way ANOVA followed by Bonferroni post-test: aAb-IL6-positive (HFD) versus sham-control (HFD), $*P = 0.05$; $***P < 0.001$. Glucose tolerance was compared by a paired Student's *t*-test: aAb-IL6-positive (HFD) versus sham-control (HFD), $*P = 0.05$. Values are expressed as average \pm s.e.m., with $n = 7-11$, see Materials and Methods for further details.

pathology (Lernmark 2001). For unknown reasons, humans may 'spontaneously' produce high amounts of neutralizing aAb to a number of growth factors and cytokines, and following the immunological breach the plasma levels of these aAb remain constant over time (Hansen *et al.* 1993, Galle *et al.* 2004b). Natural cytokine aAb are found in healthy blood donors (Bendtzen *et al.* 1998) and are readily detectable in pharmaceutically prepared pooled normal IgG (Svenson *et al.* 1993, 1998, Hansen *et al.* 1995, Ross *et al.* 1995, 1997). Above a certain threshold, aAb may render the individual deficient of the given growth factor or cytokine, however, in most cases, without overt clinical manifestations (Ross *et al.* 1997, Bendtzen *et al.* 1998, Svenson *et al.* 1998). Exceptions are: 1) granulocyte-macrophage colony-stimulating factor aAb that plays a role in pulmonary alveolar proteinosis (Uchida *et al.* 2007), 2) interferon- γ aAbs that lead to susceptibility to certain infections (Madariaga *et al.* 1998, Hoflich *et al.* 2004, Kampmann *et al.* 2005, Patel *et al.* 2005), and 3) aAb-IL6 that are being associated with recurrent staphylococcal cellulitis and subcutaneous abscesses (Puel *et al.* 2008).

We have previously shown that aAb-IL6 is detectable in plasma from 10% of Danish blood donors and correspondingly in normal Ig preparations, and that 1% of these blood donors have levels ($B/T > 0.40$) that cause an impairment of the IL6 signaling *in vivo* (Galle *et al.* 2004b). Very high levels of aAb-IL6 ($B/T > 0.95$) are present in plasma from 0.1% of the

blood donors (Galle *et al.* 2004b), and in mice, such antibody levels are able to blunt the acute phase response to turpentine injection resulting in a functional 'knockout' at all plasma IL6 levels (Galle *et al.* 2004a). The blood donors who carry aAb-IL6 display no signs of any other autoimmune phenomena, and no overt clinical manifestations have previously been reported in people with such polyclonal antibodies, the nature of which for the major part is of IgG1 origin (Hansen *et al.* 2007). The binding of IL6 is to the Fab fragments of the IgG and with extremely high affinity with K_d in the picomolar range (Hansen *et al.* 1993, 1995, 2007). This high-affinity binding of IL6 to IgG is distinct from the binding of IL6 to the soluble forms of the IL6R and the GP130. Both circulate in nanomolar concentrations, with the latter having only nanomolar affinity to IL6 (Richards *et al.* 2006). Hence, 25–50% of normal picomolar concentrations of IL6 are expected to be present as complex to soluble IL6Rs providing the basis of the IL6 trans-signaling, i.e. IL6 signaling on GP130-positive cells that express insignificant levels of membrane-anchored IL6R (Galle *et al.* 2004b, Rose-John *et al.* 2006).

Based on the observation that *Il6* gene deletion in mice may eventually lead to obesity and hyperglycemia (Wallenius *et al.* 2002, Di Gregorio *et al.* 2004), and that patients with rheumatoid arthritis who are treated with IL6R antibodies develop hypercholesterolemia and hyperglycemia (Nishimoto

Table 2 Fasting plasma levels of leptin and insulin in interleukin-6 (IL6) autoantibody positive and negative mice on normal or high-fat diet (HFD). The effects of aAb-IL6 on the plasma (p-) levels of leptin and insulin. Mice carrying IL6 autoantibodies (aAb-IL6-positive: $0.40 < B/T < 0.80$) were compared with sham-vaccinated (sham-control) mice (aAb-IL6: $B/T = 0$). Subsequently, the mice were exposed to a normal chow or high-fat diet (HFD). Data, mean \pm s.e.m. ($n = 7-11$), were evaluated by a paired Student's *t*-test. aAb-IL6-positive (HFD) versus sham-control (HFD)

	Sham-control		aAb-IL6-positive	
	Chow ($n = 8$)	HFD ($n = 9$)	Chow ($n = 11$)	HFD ($n = 7$)
p-Leptin (pM)	299 \pm 155	1241 \pm 375	175 \pm 98	4842 \pm 1538*
p-Insulin (pM)	66 \pm 19	160 \pm 30	98 \pm 35	299 \pm 77

* $P < 0.05$.

Table 3 Expression of liver enzymes in interleukin-6 (IL6) autoantibody positive and negative mice on normal or high-fat diet (HFD). The effects of aAb-IL6 on the mRNA levels of liver fibrinogen, haptoglobin, phosphoenolpyruvate carboxykinase-1 (*Pepck*), glucose 6-phosphatase (*Glu-6-Pase*), and liver protein levels of STAT3 including phosphorylated fraction (STAT3-P). Mice were vaccinated to induce low levels of IL6 autoantibodies (aAb-IL6-positive: $0.40 < B/T < 0.80$) or sham vaccinated (aAb-IL6: $B/T = 0$, sham-control). Subsequently, the mice were exposed to a normal chow or high-fat diet (HFD). Values are expressed as mean \pm s.e.m. ($n = 7-11$)

	Sham-control		aAb-IL6-positive	
	Chow ($n = 8$)	HFD ($n = 9$)	Chow ($n = 11$)	HFD ($n = 9$)
Fibrinogen (au)	2.1 ± 0.4	1.9 ± 0.2	$1.0 \pm 0.1^\dagger$	$1.0 \pm 0.1^\ddagger$
Haptoglobin (au)	1.4 ± 0.2	1.8 ± 0.1	1.0 ± 0.1	$1.2 \pm 0.8^\S$
<i>Pepck</i> (au)	1.7 ± 0.2	1.1 ± 0.1	1.8 ± 0.2	1.3 ± 0.2
<i>Glu-6-Pase</i> (au)	1.4 ± 0.3	0.5 ± 0.1	2.0 ± 0.3	$1.3 \pm 0.4^\parallel$
STAT3 (au)	1.0 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	$1.0 \pm 0.1^\#$
STAT3-P/STAT3	1.0 ± 0.1	0.8 ± 0.1	$0.5 \pm 0.1^*$	0.4 ± 0.1^a

au denotes arbitrary units. Data were compared by a paired Student's *t*-test. aAb-IL6-positive (chow) versus sham-control (chow): $*P < 0.001$; $^\dagger P = 0.02$; aAb-IL6-positive (HFD) versus sham-control (HFD): $^\ddagger P < 0.001$; $^\S P < 0.009$; $^\parallel P = 0.05$; $^\# P = 0.04$; $^a P = 0.01$.

et al. 2003), we studied the role of aAb-IL6 in type 2 diabetes. The present report of increased plasma binding activity of aAb-IL6 in a subset of type 2 diabetic patients is the first to suggest a potential pathogenic impact of aAb-IL6 in humans, except for a case report of recurrent staphylococcal cellulitis and s.c. abscesses (Puel *et al.* 2008). To further explore the possible role of aAb-IL6 as a risk factor in the pathogenesis of diabetes, we examined the metabolic implications of different plasma levels of aAb-IL6 induced in male DBA/1 mice.

Short-term metabolic effects of IL6 have been thoroughly investigated in rodents, and these results show that raising the plasma level of IL6 in obese rats for 5–10 days impairs hepatic insulin sensitivity (Klover *et al.* 2003, Cai *et al.* 2005), whereas abolishing IL6 for 3 days improves liver insulin sensitivity (Klover *et al.* 2005). On the other hand, *Il6* gene-deficient mice develop mature-onset obesity and glucose intolerance (Wallenius *et al.* 2002) preceded by an impaired ability to exercise and to oxidize fatty acids (Faldt *et al.* 2004). Likewise, these animals have a diminished activity of AMP-activated protein kinase (AMPK) in adipose and skeletal muscle tissues (Kelly *et al.* 2004).

Here, we report that the phenotype of the mice vaccinated to induce plasma aAb-IL6 with $B/T > 0.95$ was very similar to that of *Il6* gene-deficient mice (Wallenius *et al.* 2002). Notably, the *Il6* gene-deficient mice develop glucose intolerance following the onset of obesity, whereas the mice with 'knockout levels' of aAb-IL6 develop glucose intolerance without concomitant obesity and at an earlier age. It should be emphasized that the specific phenotype of the *Il6* gene-deficient mice may also be due to lack of IL6 during embryogenesis and early development, whereas IL6 signaling is not disturbed before adolescence in the present model.

In the liver, STAT3 regulates glucose production by suppressing rate-limiting enzymes of the gluconeogenic pathway. Moreover, an IL6–STAT3 signaling pathway is activated in the liver in response to insulin action in the brain (Inoue *et al.* 2004, 2006). The mechanism involves activation of afferent nerves that innervate Kupffer cells,

which in a paracrine manner releases IL6. The binding of IL6 to receptors on hepatocytes results in phosphorylation of STAT3, which down-regulates the expression of *Glu-6-Pase* and *PEPCK* (Inoue *et al.* 2004, 2006). In accordance, in mice with aAb-IL6 levels pertinent to the human situation we showed a decrease in STAT3 phosphorylation and an increase in expression of *Glu-6-Pase* and *PEPCK* in the liver in response to an s.c. insulin challenge (Table 3). We did not find any effects of aAb-IL6 on insulin-stimulated phosphorylation of AKT, IRS1, or IR in the liver or the soleus, gastrocnemius, and EDL muscle.

Several pieces of evidence suggest that the pathological potential of aAb-IL6 depends on plasma levels, age (time of exposure to the antibodies), and an environmental factor such as diet. First, the mice with knockout plasma levels of aAb-IL6 display glucose intolerance following the loss of immunological tolerance, whereas the mice with plasma levels of aAb-IL6 pertinent to the human situation develop glucose intolerance only when challenged with a HFD. Secondly, the mice with 'human levels' of aAb-IL6 did not develop an abnormal metabolic phenotype until after 12–15 weeks of aAb-IL6 presence, whereas the mice with the high plasma levels of aAb-IL6 develop impaired metabolic homeostasis after only 4 weeks. Thirdly, in the logistic regression analysis of the human data we demonstrated that patients with plasma levels of aAb-IL6, which in mice impairs IL6 signaling, have increased risk of developing type 2 diabetes with age as compared with persons without antibodies. Accordingly, the control subjects with these levels of aAb-IL6 were younger than the median age of the respective cohorts, whereas the type 2 diabetic patients with similar levels of aAb-IL6 were in general older than the median age (Fig. 1).

In conclusion, the prevalence of plasma aAb-IL6 at levels impairing IL6 action *in vivo* was increased 2.5-fold in patients with type 2 diabetes. In mice, aAb-IL6 levels pertinent to the human situation caused obesity and impaired glucose regulation. These findings suggest that aAb-IL6 are

pathogenic in a small subset of patients with type 2 diabetes and a risk factor for development of hyperglycemia in 1% of Danish blood donors on a westernized diet. The identification of a potential novel diabetogenic immunological mechanism raises the question whether aAb-IL6 should be added to the known predictors of type 2 diabetes when screening at risk people. Also, it should be considered if re-establishment of immunological tolerance to endogenous IL6 might be a viable anti-hyperglycemic therapeutic approach in a subset of patients with type 2 diabetes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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