Lack of Invariant Natural Killer T Cells Affects Lipid Metabolism in Adipose Tissue of Diet-Induced Obese Mice

Daniela Stroothoff,* Anna M. Lundberg,* Hanna E. Agardh, Daniel F.J. Ketelhuth, Gabrielle Paulsson-Berne, Peter Arner, Göran K. Hansson, Norbert Gerdes

Objective—Obesity promotes a chronic inflammatory condition in adipose tissue (AT). Impairment of insulin sensitivity coincides with infiltration of T cells into AT in early stages of obesity, when macrophages are not yet present. Here, we examine the role of invariant natural killer T (iNKT) cells, a subtype of T cells activated by lipid antigens, on glucose and lipid metabolism in obesity.

Approach and Results—Jα18−/− mice, specifically lacking iNKT cells, and wild-type mice consumed a chow or high-fat diet for 10 weeks. One third of all T lymphocytes in the liver of wild-type mice were iNKT cells, whereas few were detected in AT. Diet-induced obesity increased blood glucose in both genotypes of mice, whereas glucose tolerance test revealed similar kinetics of glucose clearance in Jα18−/− and wild-type mice. Under obese conditions, expression of inflammatory cytokines in AT did not differ between the groups, although the number of T cells and macrophages was lower in Jα18−/− mice. Nonetheless, AT homeostasis in Jα18−/− mice was altered evidenced by lower AT weight, smaller adipocytes, accelerated lipogenesis, increased expression of hormone-sensitive lipase, and accelerated basal lipolysis.

Conclusions—inNKT cells do not affect glucose clearance but rather modulate lipid metabolism in both liver and AT. Only few iNKT cells are found in AT under lean and obese conditions, suggesting that their effects on lipid metabolism are mainly mediated in the liver, their primary host organ. (Arterioscler Thromb Vasc Biol. 2013;33:1189-1196.)

Key Words: immune system ■ leukocytes ■ lipases ■ NKT cells ■ obesity

Natural killer T (NKT) cells are leukocytes that combine characteristics from both natural killer cells and T lymphocytes. In contrast to major histocompatibility complex class I and major histocompatibility complex II–restricted T cells, NKT cells recognize glycolipids presented by CD1d molecules. Two types of NKT cells can be distinguished based on their T cell receptors. Type I NKT cells express the invariant T cell receptor chain (Vα14-Vβ18 [mouse], Vα24-Vβ18 [human]), and they are restricted to recognize antigen presented by the major histocompatibility complex–related CD1d molecule.1 They account for up to 30% of the liver lymphocytes in mice and are the focus of our study.2 Type II NKT cells on the contrary may have several different Vα chains.1

NKT cells were ascribed a crucial role in numerous pathologies and conditions, such as viral and bacterial infections, autoimmune diseases, atherosclerosis, and cancer; however, little is known about their role in adipose tissue (AT) inflammation and metabolic disorders. Obesity is associated with a chronic inflammatory condition in the AT, characterized by infiltration of macrophages and, as recently discovered, T cells.3–5 Studies of diet-induced obesity (DIO) in mice have demonstrated impairment of insulin sensitivity coinciding with infiltration of T cells into AT in early stages of obesity, when macrophages were not yet present.6 Although T cells might contribute to DIO-driven inflammation, they do not suffice to cause insulin resistance in hyperlipidemic, but lean, mice.3 However, several proinflammatory cytokines produced by T cells impact on metabolic pathways in AT, including tumor necrosis factor-α, interleukin-6, and interferon-γ.3,7 The liver has a central role in both glucose and lipid metabolism. Interestingly, it also harbors the main reservoir of NKT cells in the body, and these cells may exert a pronounced effect on lipid metabolism. In the current study, we examined the impact of invariant NKT (iNKT) cells on glucose and lipid metabolism in liver and AT.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Received on: December 30, 2012; final version accepted on: February 26, 2013.

From the Department of Medicine, Center for Molecular Medicine, Karolinska Institute, Karolinska University Hospital, Stockholm, Sweden (D.S., A.M.L., H.E.A., D.F.J.K., G.P.-B., G.K.H., N.G.); Department of Medicine, Karolinska University Hospital Huddinge, Karolinska Institute, Stockholm, Sweden (P.A.); Institute for Cardiovascular Prevention (IPEK), Ludwig-Maximilians-University (LMU), Munich, Germany (N.G.); and Department of Medical Biochemistry, Academic Medical Center (AMC), University of Amsterdam, Amsterdam, The Netherlands (N.G.).

*These authors contributed equally to this work.

The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBHA.112.301105/-/DC1.

Correspondence to Norbert Gerdes, Institute for Cardiovascular Prevention (IPEK), Pettenkoferstrasse 9, Ludwig-Maximilians University Munich, 80336 Munich, Germany. E-mail norbert.gerdes@med.uni-muenchen.de

© 2013 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org DOI: 10.1161/ATVBHA.112.301105
Results
iNKT Cells Are a Major Lymphocyte Population in Liver, but Not in AT
Staining with α-GalCer–loaded CD1d-Ig dimer protein (DimerX) was used in combination with anti-NK1.1 to identify iNKT cells in AT and liver. Flow cytometric analysis of liver leukocytes confirmed specific deficiency of iNKT cells in Ja18−/− mice (<0.2%), whereas this population represented 32% of all CD4+ T cells in the liver of wild-type mice (Figure 1A and 1B). In contrast to liver, AT of wild-type as well as Ja18−/− mice contained very few or no iNKT cells, respectively (Figure 1D and 1E). Furthermore, real-time polymerase chain reaction analysis of T-cell receptor mRNA of liver confirmed that AT contained <1000 iNKT cells per gram tissue, whereas this number was ≈200-fold higher in the liver (Figure 1C and 1F). Additionally, real-time polymerase chain reaction analysis for the specific Vo14Ja18 T-cell receptor mRNA of liver and AT confirmed that iNKT cells are not present in Ja18−/− mice (Figure IA and IB in the online-only Data Supplement).

Absence of iNKT Cells Does Not Influence Glucose Clearance
To examine the impact of iNKT cell deficiency on glucose metabolism, intraperitoneal glucose tolerance test was performed on wild-type and Ja18−/− mice that consumed high-fat diet (HFD) or chow diet for 10 weeks. Although fasting glucose did not differ between the groups (Figure 2A), Ja18−/− mice had lower body and fat weight, irrespective of the diet (Figure 3A and 3B). Intraperitoneal glucose tolerance test revealed that lack of iNKT cells does not affect blood glucose clearance after consuming chow diet. DIO impaired blood glucose clearance similarly in Ja18−/− and wild-type mice (Figure 2B and 2C). Similar results were obtained when mice were fasted for only 6 hours before performing intraperitoneal glucose tolerance test (Figure 2D and 2E). In addition, fasting insulin levels in the circulation were lower in Ja18−/− than in wild-type mice fed with a chow diet but did not differ significantly in mice fed with HFD (Figure 2D). We also measured the kinetics of insulin release after an IP glucose challenge. Interestingly, wild-type mice required much higher insulin levels to maintain glycemic control compared with Ja18−/− mice (Figure 2E). Accordingly, calculation of the Homeostasis Model of Assessment–Insulin Resistance (HOMA-IR) confirmed that insulin sensitivity was better preserved in Ja18−/− mice compared with controls (Figure 2F), which also was associated with enhanced expression of insulin-regulated glucose transporters (Glut2 and -4) in liver and AT, respectively (Figure 2G).

Figure 1. Few invariant natural killer T (iNKT) cells are present in adipose tissue (AT) flow cytometric analysis of iNKT cells in liver (A–C) and AT (D–E) from wild-type (A, D) and Ja18−/− mice (B, E) fed high-fat diet (HFD) for 10 to 14 weeks. Representative plots (A, B, D, and E) and absolute numbers (C, F) are shown. Cells in the gate are defined as percentage of single, live CD19−, CD3+, CD4+, DimerX+, NK1.1+ lymphocytes. Absolute number of iNKT cells were analyzed in individual mice. Liver: n=8; AT: n=6 or 8, wild-type and Ja18−/−, respectively. Data are presented as mean±SEM. *P<0.01, ***P<0.001.
iNKT Cell–Deficient Mice Have Less Fat, Smaller Adipocytes, but Similar Lean Body Mass

The diminished body weight and AT mass (Figure 3A and 3B) resulted from both lower weight at study start and diminished weight gain over the period of HFD consumption (Figure IIC and IID in the online-only Data Supplement). These data led us to investigate body composition in HFD-fed mice. Indeed, the reduced body fat weight in Jα18−/− mice was confirmed by MRI and attributable to both smaller visceral and subcutaneous fat depots, whereas lean body mass was unchanged (Figure 3C–3G). This decrease in body fat suggested differences in AT homeostasis in Jα18−/− mice. Indeed, adipocyte volume, calculated from the cell diameter, was decreased in Jα18−/− mice, whereas the number of adipocytes was stable (Figure 3H and 3I). This change in adipocyte size was observed under both dietary conditions but paralleled the reduced AT weight and overall body weight in Jα18−/− mice, which appeared as a result of reduced food intake (Figure 3J). Of note, most parameters (eg, body weight, AT weight, glucose control) were confirmed in female mice (Figure IVA–IVD).

Increased Lipogenesis in iNKT Cell–Deficient Mice Is Counterbalanced by Elevated Lipase Expression and Basal Lipolysis

Lipogenesis, determined by incorporation of radio-labeled glucose into lipids of the adipocytes, was increased in iNKT cell–deficient mice compared with wild-type mice with and without insulin stimulation. Notably, in both strains of mice, lipogenesis was lower after consuming HFD when compared with those fed with chow diet (Figure 4A and 4B). Increased lipogenesis in AT of Jα18−/− mice did not result in elevated plasma triglyceride or cholesterol concentrations (Figure 4C and 4D) on chow diet or HFD. Considering this discrepancy, we analyzed mRNA for lipoprotein lipase (LPL) and hormone-sensitive lipase (HSL), the 2 major enzymes involved in degradation and hydrolyzation of triglycerides in AT. Indeed, quantitative polymerase chain reaction revealed increased expression of LPL (liver) and Glut4 (adipose tissue [AT]). Open symbols, wild-type mice; filled symbols, Jα18−/− mice. N=7 to 17 mice per group. Data are presented as mean±SEM, *P<0.05, **P<0.01.

Figure 2. Absence of invariant natural killer T (iNKT) cells does not influence glucose clearance but improves insulin sensitivity. A, Fasting glucose was measured in wild-type and Jα18−/− mice that consumed either a chow diet or high-fat diet (HFD) for 10 weeks. B, Intraperitoneal glucose tolerance test was performed on overnight-fasted mice by injecting glucose (1g/kg; IP), and (C) area under the curve (AUC) was calculated. Concentration of glucose and (D) leptin, and (E) adiponectin were measured in plasma. G, mRNA expression of Glut2 (liver) and Glut4 (AT). Open symbols, wild-type mice; filled symbols, Jα18−/− mice. N=7 to 17 mice per group. Data are presented as mean±SEM, *P<0.05, **P<0.01.
in $J\alpha 18^{-/-}$ mice on HFD, whereas a similar tendency was seen in the mice on chow diet (Figure 4F). Additional hormonal stimulation of lipolysis did not differ between wild-type and $J\alpha 18^{-/-}$ mice (Figure IIIA and IIIB in the online-only Data Supplement). Similar results were obtained in female mice (Figure IVE and IVF in the online-only Data Supplement).
Although iNKT cell deficiency influences lipid metabolism in AT, no change in expression of genes involved in triglyceride formation (Dgat) or cholesterol synthesis (HMG-CoA reductase) was observed in the liver (Figure 4G and 4H). The latter observation is consistent with the unaltered cholesterol concentrations measured in the plasma (Figure 4D).

Moreover, mRNA expression of hepatic lipase and MTTP was unaffected in the liver of $J_{a18}^{-/-}$ mice. As expected, LIGHT mRNA was decreased in $J_{a18}^{-/-}$ mice in both dietary conditions (Figure 4G and 4H), probably because of the absence of LIGHT-expressing iNKT cells in the liver. Finally, levels of free fatty acids increased in mice of both genotypes but did not differ between wild-type mice and those deficient for iNKT cells, suggesting operation of additional mechanism of free fatty acids catabolism (Figure 4I).

### Lack of iNKT Cells Does Not Influence Expression of Inflammatory Genes in AT of DIO Mice

Quantitative polymerase chain reaction analysis revealed a diminished inflammatory response in the liver of $J_{a18}^{-/-}$ compared with wild-type mice after consuming either chow diet or HFD (Figure VA–VC in the online-only Data Supplement; Figure 5E). Absolute numbers of leukocytes and their major subsets, such as macrophages and T cells, were decreased in the liver of $J_{a18}^{-/-}$ mice compared with wild-type controls, both when calculating total cells per organ (Figure 5B–5D) or correcting for organ weight (data not shown). In addition, under normal dietary conditions, $J_{a18}^{-/-}$ mice displayed lower expression in AT of some inflammatory transcripts (eg, CD68 and tumor necrosis factor-$\alpha$), indicating a role for iNKT cells in immune homeostasis under physiological conditions (Figure VD and VF in the online-only Data Supplement). Although we observed lower numbers of inflammatory cells residing in AT of $J_{a18}^{-/-}$ mice consuming HFD (Figure 5F–5I), no difference in expression of any of the immune-related transcripts was found in the AT, suggesting that these cells do not directly influence obesity-induced AT inflammation (Figure 5J; Figure VE in the online-only Data Supplement). Of note, total numbers of leukocytes and their subsets were considerably higher in liver than in AT (Figure 5) mirroring the distribution of iNKT cells (Figure 1C and 1F), and suggesting that lack of these cells may influence processes in their primary host organ more potently than in AT.

### Discussion

The results of the present study show that iNKT cells modulate lipid and insulin metabolism without directly affecting plasma glucose clearance. Furthermore, they show that metabolically active iNKT cells reside in much greater numbers in the liver rather than in AT. Using a well-defined combination of markers for iNKT cells, we found only few of them in AT, despite repeated analyses of T cell subpopulations. However, we cannot exclude that these cells, although small in number, may actively modulate adipocyte metabolism. Our data are contrasted by groups reporting large numbers of iNKT cells in AT$^{6,10}$ but corroborate other studies that failed to detect iNKT cells in AT$^{11,12}$. Although we cannot provide a final explanation for this controversy, differences in preparation or experimental protocols are most likely. For instance, subtle differences in the genetic background of the mouse strains under study, as well as microbiological conditions in the different animal facilities, could influence the metabolic and immune state of the animals.$^{13}$ Furthermore, analytical methods differ with regard to flow cytometric staining procedures and reagents (eg, we
used aGalCer-loaded CD1d dimer, whereas others used CD1d tetramers), and the present study is the only one using mRNA analysis to validate conclusions from flow cytometric data. In addition, we found considerable binding of the CD1d dimer to dead cells, which were excluded from our quantitative analysis (data not shown). Finally, HFD may change the activation status of the iNKT cells and influence the expression level of their specific T cell receptors, thus compromising conclusions when using CD1d multimers.

The impairment of glucose clearance on HFD was similar in mice that lack iNKT cells as in wild-type mice. This is in line with a recent published article by Mantell et al.11 but contrasts the findings of Ohmura et al.12 We observed that lack of iNKT cells results in better insulin sensitivity reflected by lower insulin levels and elevated basal expression of insulinsensitive glucose transporters in liver and AT. However, this increased sensitivity did not result in improved glucose clearance, probably as a result of lower insulin level secreted by the mice lacking iNKT cells. We further investigated the cross-talk between glucose and lipid metabolism using the mice devoid of iNKT cells attributable to targeted deletion of the iNKT cell-specific Jα18 T cell receptor gene. Our study revealed that iNKT cells mainly reside in the liver, and that their absence leads to the following: (1) diminished inflammatory response in liver; (2) increased lipogenesis, which is counterbalanced by increased HSL and LPL expression and increased lipolysis; and (3) decreased adipocyte volume.

Although a possible malignant role in glucose metabolism was recently proposed,13 our data rather suggest that iNKT cells residing in the liver influence AT homeostasis. We observed that mice lacking iNKT cells have lower body weight, implying that AT homeostasis may differ compared to wild-type mice. Indeed, adipocytes from Jα18−/− mice were smaller than those of control mice, whereas their number did not change. In addition, MRI analysis revealed similar lean body mass in the 2 groups, whereas Jα18−/− mice displayed a reduction of total body fat that was attributable to both the visceral and subcutaneous fat compartment.

Several factors define AT homeostasis, the balance of triglyceride synthesis (lipogenesis), and breakdown of triglyceride (lipolysis) in adipocytes on one hand and uptake of triglycerides from the circulation on the other. The dynamics of adipose lipid turnover seem to play a major role in metabolic disease.14 Indeed, although smaller in volume, adipocytes from Jα18−/− mice have an increased turnover of triglycerides from the circulation indicated by increased expression of LPL in AT. Furthermore, we detected elevated expression of HSL in AT. Of note, LPL and HSL expression and activity can be regulated by different cytokines. Both tumor necrosis factor-α, which was decreased in AT and liver of Jα18−/− mice fed with normal chow diet, and interferon-γ, which was decreased in the liver, are known to inhibit LPL and HSL.15,16 HSL is the key enzyme initiating adipocyte lipolysis,17,18 and we could demonstrate that the increased HSL expression in Jα18−/− mice is accompanied by increased (basal) lipolysis. The increased lipolysis potentially promotes release of free fatty acids that is counterbalanced by improved lipogenesis overall, resulting in plasma triglyceride concentrations that are similar to that of wild-type mice. The discrepancy between an increased lipolysis and unchanged free fatty acids levels in the Jα18−/− mice may also be a consequence of enhanced catabolic processes that use circulating fatty acids and can also result from increased insulin sensitivity.21

There is a strong connection between obesity and fatty liver disease, also termed nonalcoholic steatohepatitis,22 indicating an indirect impact of cytokines/adipokines released in the liver or AT. Indeed, studies observed reduced NKT cell number in leptin-deficient ob/ob mice,23 and adoptive transfer of NKT cells into ob/ob mice positively influenced glucose metabolism, resulting in decreased liver fat content.24 Although the mechanisms underlying these findings are not fully understood, we studied the connection between liver and AT under obese conditions from another perspective by depleting iNKT cells, which resulted in decreased leptin secretion from AT. Indeed, iNKT cells may directly affect leptin release, but it is also possible that the morphological changes (ie, smaller adipocytes and lower fat mass) lead to reduced leptin secretion.

NKT cells are a diverse population of T cells, classification of which was refined over the past years. They have been described in AT as CD3+CD14+1 T cells.2 Nowadays, it is widely accepted that the characterization of NKT cells only based on NK1.1 is not sufficient.1 We used the combination of α-GalCer-loaded CD1d dimer (DimerX) and NK1.1 to identify iNKT cells and found only few iNKT cells in AT of wild-type mice. Of note, a minor DimerX NK1.1+ T cell population could be detected in Jα18−/− mice. These cells may represent type II NKT cells, the role of which in AT was recently studied by Satoh et al.12 who corroborate our findings of minor presence and functional involvement of iNKT cells in AT inflammation during DIO. Another explanation is that these DimerX NK1.1+CD3+CD4+ cells are mucosal-associated NKT cells, so far only detected in the liver, blood, spleen, lymph nodes, and bone marrow.25 However, our study focused on the invariant CD1d-restricted iNKT cells, whereas other subsets were not further investigated.

Furthermore, we could not identify any changes in expression of inflammatory cytokines in AT of obese mice lacking iNKT cells compared with wild-type mice. Corroborating the pivotal role of iNKT cells in the liver, Jα18−/− mice displayed a less inflammatory phenotype compared with the wild-type mice, evidenced by reduced CD3, interferon-γ, and LIGHT expression evoked by the removal of a major, liver-resident T lymphocyte population in these mice. Interestingly, LIGHT was previously shown expressed by hepatic iNKT cells and modulates liver metabolism.26,27 Although in our study, iNKT cell deficiency reduced cellular infiltrate in both liver and AT under obese conditions, transcripts of effector cytokines were lower only in the liver, suggesting that the reduced pathology is mainly mediated in this principal iNKT cell reservoir. Nonetheless, these changes in the liver may likely lead to a healthier AT phenotype and overall preserved insulin sensitivity. In turn, this enhanced insulin sensitivity in iNKT cell-deficient mice may promote lipogenesis because this process is tightly regulated by insulin.

Our findings were sex-independent because we observed very similar results in female mice. However, male mice, which do not have pronounced hormonal fluctuations and develop more rapid obesity, are most widely used for studying
Hence, our study focused on the HFD response in male mice. Notably, our conclusions differ from those recently presented by Ohmura et al., who showed reduced numbers of inflammatory cells in AT and improved glucose control in β2 microglobulin-deficient (b2m−/−) mice. The authors attribute these changes to the absence of NKT cells in these mice, although β2 microglobulin also functions as a component of the major histocompatibility complex class I complex, which is prerequisite for functional CD8+ T cells. Because of the absence of this major T cell population, a qualified assessment of the contribution of NK and all NKT cells to AT inflammation and metabolic disturbance by this study seems questionable. The important role of CD8+ T cells in AT inflammation and glucose intolerance was initially shown by Nishimura et al. and was recently confirmed by Mantell et al. Using a mouse model that lacks CD1d, but otherwise has a complete T cell repertoire, they support our data that NKT cells do not impair glucose clearance. Here, we expand the current knowledge by using a highly specific mouse model lacking iNKT cells.

In summary, our data demonstrate a strong metabolic interaction between liver and AT. We show that depleting iNKT cells in the liver can influence AT homeostasis and lipid metabolism.

**Acknowledgments**

We thank Mikael Karlsson for providing Jα18−/− mice; and Ingrid Törnberg, Anneli Olsson, Anna-Lena Gustafsson, and Melanie Cremer for technical help; and Sahar Nikkhou Aski, Peter Damberg, and Stefan Brene for assistance with the MRI measurements.

**Sources of Funding**

This work was supported by project grants from the Swedish Research Council (grants 521-2009-4203 [project grant] and 349-2007-8703...
 Our study investigated the role of invariant natural killer T (iNKT) cells, a glycolipid-recognizing T cell subpopulation, in glucose and lipid metabolism using a mouse model specifically lacking iNKT cells. Contrary to some recent reports, we found that the majority of iNKT cells reside in the liver rather than in adipose tissue. In fact, we believe that the few iNKT cells in visceral fat will not exert a substantial contribution to adipose tissue inflammation, which is increasingly recognized as a key pathological process promoting type 2 diabetes mellitus. Nonetheless, rather than affecting glucose control, our data indicate that lack of iNKT cells promotes higher lipid turnover in adipose tissue, despite smaller adipocyte size overall associated with a more lean phenotype in diet-induced obese mice. Accordingly, therapies targeting iNKT cells may still hold promise in ameliorating metabolic diseases other than diabetes mellitus.
Lack of Invariant Natural Killer T Cells Affects Lipid Metabolism in Adipose Tissue of Diet-Induced Obese Mice
Daniela Strodthoff, Anna M. Lundberg, Hanna E. Agardh, Daniel F.J. Ketelhuth, Gabrielle Paulsson-Berne, Peter Arner, Göran K. Hansson and Norbert Gerdes

Arterioscler Thromb Vasc Biol. 2013;33:1189-1196; originally published online March 21, 2013;
doi: 10.1161/ATVBAHA.112.301105
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/33/6/1189

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2013/03/21/ATVBAHA.112.301105.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental material to Strodthoff et al.:

Lack of Invariant Natural Killer T Cells Affects Lipid Metabolism in Adipose Tissue of Diet-Induced Obese Mice

Supplementary Figure I: \(J\alpha 18^-\) mice lack V\(\alpha 14J\alpha 18\) T cell receptor-expressing iNKT cells. Mice were fed HFD for 10 weeks. Real-time PCR analysis of the iNKT cell-specific V\(\alpha 14J\alpha 18\) T cell receptor in (A) liver and (B) AT of wild-type and \(J\alpha 18^-\) mice. Analysis of NKT cell sub-populations by flow cytometry in (C) liver (D) AT. Cells in the gate are defined as percentage of single, live, CD19\(^-\), CD3\(^+\), CD4\(^+\), lymphocytes. Quantitation of flow cytometry pooled from 4-6 mice (AT 2 pools for each strain; liver 2 pools (\(J\alpha 18^-\)) and 3 pools (wild-type)) are shown. Data are presented as mean±SEM. \(*P<0.05\).
Supplementary Figure II: Absence of iNKT cells does not influence glucose clearance. (A) ipGTT was performed by injecting glucose (1g/kg; i.p.) in 6h-fasted mice fed a HFD for 10 weeks (B) AUC was calculated. Glucose concentration was analyzed in blood drawn from the tail vein. (C) Body weight and (D) body weight gain was monitored during the time of diet. Open symbol, wild-type mice; filled symbol, \( \text{J}_{\alpha}18^{+/-} \) mice. N=7-8 mice per genotype. Data are presented as mean±SEM.
Supplementary Figure III: Hormone-stimulated lipolysis is unchanged

Isolated adipocytes were incubated with isoprenaline (Iso), norepinephrine (NA), and 8-bromo-cyclic AMP (8-Br-cAMP). Lipolysis was determined by release of glycerol in mice fed a (A) chow diet or (B) HFD and is expressed as fold induction to basal lipolysis. Open bars, wild-type mice; closed bars, Jα18⁻/⁻ mice. Data are presented as mean±SEM.
Supplementary Figure IV: Absence of iNKT cells does not influence glucose clearance in female mice. (A) Body weight and (B) gonadal adipose tissue weight of female mice fed a HFD for 14 weeks. N=6, wild-type and n=5 for Jα18−/− mice. (C) ipGTT was performed on overnight-fasted mice by injecting glucose (1g/kg; i.p.) and (D) AUC was calculated. Glucose concentration was analyzed in blood drawn from
the tail vein. N=7 per genotype (E) Lipogenesis was determined in AT of mice on HFD by incorporation of radiolabelled glucose into adipocyte lipids under basal and after insulin stimulation. Lipogenesis is shown in nmol of glucose incorporated in 2 hours/gram lipid. (F) Basal lipolysis was determined by release of glycerol in mice consuming HFD. N=6, wild-type and n= 3 for Jα18⁻/⁻ mice. Open symbol, wild-type mice; filled symbol, Jα18⁻/⁻ mice. Data are presented as mean±SEM. *P<0.05, **P<0.01, ***P<0.001, n.s.= non significant.
Supplemental Material to Strodthoff et al.: Lack of Invariant Natural Killer T Cells Affects Lipid Metabolism in Adipose Tissue of Diet-Induced Obese Mice
Supplementary Figure V: Lack of iNKT cells reduces inflammatory status in the liver.

Transcripts of cell type-specific markers were analyzed by RT-PCR in (A, B) liver and (D,E) AT and transcript of cytokines were analyzed in (C) liver and (F) AT from mice consuming (A,C,D,F) chow diet or (B,E) HFD. Expression was normalized to Hprt; open bars, wild-type; filled bars, Jα18⁻/⁻ mice. n=8-11 for all. Data are presented as mean±SEM. *P<0.05, **P<0.01, ***P<0.001.
Material and Methods to Strodthoff et al.:

Lack of Invariant Natural Killer T Cells Affects Lipid Metabolism in Adipose Tissue of Diet-Induced Obese Mice

Animal experiments

Five to 8 weeks old Jα18Δ– mice, which have a non-functional Vα14Jα18 T cell receptor gene and consequently lack iNKT cells, and C57BL/6 wild-type mice were fed a high-fat diet (HFD; 34.9% fat, Altromin, Lage, Germany) or normal chow diet for 10-14 weeks. Intraperitoneal glucose tolerance test (ipGTT) was performed on overnight (i.e., 12-14h) fasted mice by injecting glucose (1g/kg body weight; i.p.) and monitoring glucose concentration (Abbott Scandinavia AB, Solna, Sweden) in blood drawn from the tail vein. HOMA-IR was calculated as following: fasting glucose (mg/dl) x fasting insulin (mU/l) / 405. Male mice were used if not indicated otherwise.

All animal experiments were approved by the Stockholm regional board for animal experimentation.

Tissue processing

Overnight-fasted mice were euthanized and blood was collected in EDTA tubes before perfusion of the arterial tree with PBS. Tissue was harvested and kept in cold PBS or was immediately frozen at -80°C (for mRNA analysis) until further processing. Where applicable, whole body weight, liver and AT weight was determined immediately. AT weight of one gonadal fat pad was measured.
**Plasma analysis**

Blood was centrifuged and plasma collected and aliquoted. Triglyceride and cholesterol were measured by using an enzymatic colorimetric method (Randox, Crumlin, UK). NEFA (Wako Chemicals GmbH, Neuss, Germany), glycerol (Sigma-Aldrich, St. Louis, USA), Insulin (Mercodia, Uppsala, Sweden and Crystal Chem INC., Illinois, USA), adiponectin (R&D Systems, Minneapolis, USA), and leptin (Peprotech, Rocky Hill, USA) were measured according to the protocols provided by the manufacturers.

**Flow cytometry**

Hepatic mononuclear cells were prepared as described previously. Gonadal AT of the respective group (Jα18–/–, wild-type) was pooled when indicated and incubated in Krebs-Ringer buffer (KRB) containing 0.05% collagenase type I and 4% BSA (both Sigma-Aldrich, St. Louis, USA) for 1h at 37°C. The cell suspension was filtered through a nylon mesh (200µm, Sefar, Heiden, Switzerland) and centrifuged following erythrocyte lysis (ACK-lysis buffer). Following two washing steps with PBS the suspension was passed through 100µm cell strainer (BD), and resuspended in PBS. Cells from this stromal-vascular cell fraction or hepatic mononuclear cells were incubated with the following antibodies: NK1.1-PE or-FITC, CD8a-PerCp, CD4-APC-H7, CD3ε-Pacific Blue, and CD19-APC (all BD, Franklin Lakes, USA). CD1d dimers (DimerX, BD, Franklin Lakes, USA) were loaded with α-galactosylceramide (αGalCer; Larodan Fine Chemicals, Malmö, Sweden) according to the manufacturer’s instructions and pre-incubated with FITC- or PE-conjugated rat anti-mouse IgG1 (BD, Franklin Lakes, USA). Dead cells were excluded employing the Live/Dead® fixable aqua dead cell stain kit (Invitrogen, Carlsbad, USA). CountBright™ Absolute Counting Beads (Invitrogen) were used to determine...
absolute cell number. Samples were fixed in 2% formaldehyde in PBS and analyzed in a CyanADP flow cytometer (Beckman Coulter, Miami, USA).

**Adipocyte cell size and cell number**

The diameter of 100 isolated adipocytes per sample was determined by microscopy. The mean adipocyte volume was calculated according to Hirsch and Gallian and sagittal diameter was used. Five hundred mg AT was used to determine adipocyte size, lipogenesis, and lipolysis. AT from Jα18−/− mice was pooled in groups of 2-3 mice to compensate small tissue sample size. Adipocyte number of 8 mice per genotype was calculated as total fat pad weight in gram divided by the fat cell weight times 10000.

**Lipogenesis**

Lipogenesis was analyzed as described in detail elsewhere. In brief, isolated fat cells were incubated for 2 h at 37°C in a concentration of 2% (v/v) in KRB buffer (pH 7.4) containing albumin (20 mg/ml), [3-3H] glucose (5x10⁶ dpm/ml), unlabelled glucose (1 μM) and human insulin at different concentrations (10⁻¹⁵ -10⁻⁶ M). Incubations were stopped by rapidly chilling to 4°C and adding H₂SO₄. The incorporation of radiolabelled glucose into adipocyte lipids was determined in a scintillator. Lipogenesis was expressed as nmol of glucose incorporated/2 hours/g TG.

**Lipolysis**

Glycerol release was used as an index of lipolysis. Adipocytes were incubated for 2 hours at 37°C in a shaking bath in KRB containing 2% BSA, 1 mg/ml glucose; isoprenaline (Iso) at 10⁻⁹-10⁻⁵ M, norepinephrine (NA) at 10⁻¹²-10⁻⁴ M, and 8-bromo-cyclic AMP (8-Br-cAMP) at 10⁻³ M. The total lipid content was measured.
gravimetrically after extraction with heptane. At the end of the incubation, a cell-free aliquot of the incubation medium was removed for glycerol determination by a bioluminescence method. Lipolysis was expressed as the glycerol concentration per lipid weight of the incubated adipocytes. Hormone induced lipolysis was expressed as fold induction compared to basal lipolysis.

**RNA isolation and quantitative PCR**

Total RNA was isolated from liver and gonadal AT using the RNeasy Lipid Mini kit (Qiagen, Hilden, Germany) and reverse transcription was performed with Superscript-II (Invitrogen, Carlsbad, USA) and random hexamers. cDNA was amplified by RT-PCR using primers and probes (obtained as “assays on demand” from Applied Biosystems, Carlsbad, USA) for CD3, CD68, IFNγ, TNFα, IL-6, lipoprotein lipase (Lpl), hormone sensitive lipase (Hsl), hepatic lipase, diglyceride acyltransferase (Dgat), 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA), hepatic lipase, TNF ligand superfamily member 14 (LIGHT), microsomal triglyceride transfer protein (Mttp) and hypoxanthine-guanine phosphoribosyltransferase (Hprt).

Vα14Jα18 TCR was amplified, as also described previously,6 7 using the following primers; Forward primer: 5'-TGG ATG ACA CTG CCA CCT ACAT-3'; Reverse primer: 5'-TCC AAA ATG CAG CCT CCC TA -3'; The probe was newly designed: 6FAM-5'-CAG CCT CCC TAA GGC TGA ACC TCT-3'-TAMRA.

All amplification and analyses were performed on an ABI 7900HT Sequence Detector (Applied Biosystems). Data were calculated as $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T (\text{sample}) - \Delta C_T (\text{calibrator} = \text{average } C_T \text{ values of all samples within each group})$, and $\Delta C_T$ is the $C_T$ of the target gene after subtraction of the $C_T$ value for the housekeeping gene ($Hprt$).
**Food intake**

Food intake was monitored in individually-housed mice over a period of 48h and displayed as food intake [g] per mouse per day [24h].

**Magnetic resonance imaging (MRI)**

To determine the distribution of body fat magnetic resonance imaging (MRI) were performed on isoflurane-anesthetized mice. Animals were placed in a 9.4 T magnet with a bore size of 31 cm (Varian, Yarnton, UK) in a gradient system with a 12 cm inner diameter and a maximum gradient strength of 600 mT/m. The core body temperature and the respiration were monitored during scanning (SA-instruments) and body temperature was maintained at 37°C during MRI scanning with a thermostated warm air system (SA-instruments). A volume coil of the millipede design (Varian.inc) with 40 mm inner diameter and 110 mm RF-window was employed both for excitation and detection. 40-45 axial slices, from neck to tail, of 1 mm thickness with 0.5 mm gap, with a field-of-view of 40x40 mm² and a matrix size of 256x96 were acquired. The spin-echo sequence was employed with an echo time of 15 ms and one average. Preceding every excitation pulse, a 6 ms five lobe sinc pulse, achieving a selective 90° flip for water followed by a 2 ms crusher gradient of 11 G/cm was applied to saturate water, while leaving the fat signal unperturbed. Respiration gating was employed, with ten blocks of data acquired during each expiration period. A pixel counting-based determination of fat volumes was performed on two-dimensional image series analyzed using Fiji image analysis. A density factor of 0.9 g/ml was used to convert fat volumes (in ml) into fat mass (in g). Lean fat mass was calculated by body weight subtracted by total fat (BW-total fat).

**Statistics**
Results are expressed as mean ± SEM. Statistically significant differences were determined by the Mann Whitney test, and p values <0.05 were considered significant. Area under the curve (AUC) was calculated to compare differences over time.

Reference