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*J Immunol* published online 28 October 2013
http://www.jimmunol.org/content/early/2013/10/25/jimmunol.1301176

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**Supplementary Material**
http://www.jimmunol.org/content/suppl/2013/10/30/jimmunol.1301176.DC1

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Cutting Edge: IL-25 Elicits Innate Lymphoid Type 2 and Type II NKT Cells That Regulate Obesity in Mice

Emily Hams,*† Richard M. Locksley,‡ Andrew N. J. McKenzie,§ and Padraic G. Fallon*†,‡,*

The cellular composition of visceral adipose tissue (VAT) and release of cytokines by such cells within VAT has been implicated in regulating obesity and metabolic homeostasis. We show the importance of IL-25–responsive innate cells, which release the Th2 cytokine IL-13, in regulating weight and glucose homeostasis in mouse models of diet-induced obesity. Treating obese mice with IL-25 induces weight loss and improves glucose tolerance, and is associated with increased infiltration of innate lymphoid type 2 cells (ILC2), type I and type II NKT cells, eosinophils, and alternatively activated macrophages into the VAT. By depleting ILC2 in obese Rag1–/– mice, we observe exacerbated weight gain and glucose intolerance. Conversely, transferring ILC2 or type I or type II NKT cells into obese mice induces transient weight loss and stabilizes glucose homeostasis. Our data identify a mechanism whereby IL-25 eliciting IL-13–producing innate cells regulates inflammation in adipose tissue and prevents diet-induced obesity. The Journal of Immunology, 2013, 191: 000–000.

A hallmark of obesity is chronic low-grade inflammation that has a pivotal role in the progression to metabolic disorders, such as atherosclerosis and type 2 diabetes. The inflammatory cell composition of adipose tissue can profoundly influence the regulation of weight and the maintenance of metabolic homeostasis. Although there is a range of resident populations of immune cells in visceral adipose tissue (VAT), such as eosinophils, effector and memory T cells, regulatory T cells, and NKT cells, there is also a marked macrophage infiltration that is further increased in VAT of obese individuals (1). The polarization of macrophages within VAT to classically activated (CAM) or alternatively activated macrophages (AAMs) influences metabolic homeostasis; in the VAT of lean individuals, AAMs predominate, whereas obesity leads to a CAM-dominated infiltration of the VAT (2). The cells in VAT that release cytokines that initiate the polarization of macrophages to a CAM (INF-γ, IL-6, or TNF-α) or AAM (IL-4 and IL-13) profile are not fully elucidated.

Although studies into homeostatic glucose regulation have outlined the importance of AAM in promoting insulin sensitivity, eosinophils have been shown to be important in sustaining AAM in the VAT of mice on high-fat diet (HFD), by the localized release of IL-4 and IL-13 (3). More recently, attention has focused on the role of innate lymphoid cell (ILC) types that are present in the VAT, in particular, type 2 ILC. Interestingly, one such ILC2 population was identified in fat-associated lymphoid clusters in both mice and humans (4). A recent study in mice has eloquently identified a role for ILC2, induced in response to helminth infection or exogenous treatment with the ILC2-eliciting cytokine IL-33, in the localization of eosinophils within VAT and the local expansion of AAM (5). In this article, we show that IL-25–responsive innate cells, including IL-13–producing ILC2 and NKT cells, regulate weight and glucose homeostasis in mice on HFD via the induction and maintenance of eosinophils and AAM in the VAT.

Materials and Methods

Animals

C57BL6/J and Rag1-deficient C57BL6/J mice (Rag1–/–) were purchased from Jackson Laboratories (Bar Harbor, ME). Il17br–/– (Il17br–/–) mice were on a C57BL6/J background. Cd1d-deficient (Cd1d–/–) or IL-13-effector reporter mice (6), IL-13–deficient mice (Il3–/–) (7), and IL-17BR–deficient mice (Il17br–/–) (8) were backcrossed to C57BL6/J background in-house. Il-13eGFP (6) mice were crossed with H2Knb2 mice (8) in-house. In all studies, male mice of 7–9 wk of age were used. Animals were housed in a specific pathogen-free facility in individually ventilated and filtered cages under positive pressure. All animal experiments were performed in compliance with the Irish Medicines Board regulations and approved by the Trinity College Dublin’s BioResources ethical review board.

HFD, in vivo glucose testing, and interventions

Age-matched mice were fed an HFD (60% kcal fat; D12492; Research Diets) or control diet (10% kcal fat) for 8–16 wk as indicated. Glucose tolerance was assessed in mice fasted overnight and challenged with 2 g/kg glucose intraperitoneally.

Received for publication May 10, 2013. Accepted for publication September 24, 2013.

This work was supported by grants from Science Foundation Ireland, the National Children’s Research Centre, and the Health Research Board.

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The online version of this article contains supplemental material.

Abbreviations used in this article: AAM, alternatively activated macrophage; CAM, classically activated macrophage; α-Gal-Cer, α-galactosylceramide; HFD, high-fat diet; ILC, innate lymphoid cell; VAT, visceral adipose tissue; WT, wild-type.

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glucose i.p., and blood was analyzed by a glucose meter (Abbott Laboratories, Chicago, IL). After 8 wk on HFD, mice were injected on days 0, 2, and 4 with 0.4 µg recombinant mouse IL-25 (R&D Systems, Abingdon, U.K.), 20 µg α-galactosylceramide (α-Gal-Cer; Avanti Polar Lipids, AL), or 20 µg sulfatides (Matreya, Pleasant Gap, PA), and weight was monitored for 6 d. For neutralizing experiments, 250 µg/mouse anti-CD90.2 mAb or rat IgG2b isotype control (BioXCell) was administered i.p. every 3 d at commencement of feeding HFD.

**Flow cytometry**

VAT from lean and obese mice was mechanically shredded and incubated with 1 mg/ml collagenase D from *Claytradium histolyticum* (Roche Applied Science, West Sussex, U.K.), and a single-cell suspension was prepared. Surface marker expression was assessed by flow cytometry with data collection on a CyAn (Beckman Coulter), and data were analyzed using FlowJo software (Tree Star). IL-4 expression in the cells was identified in IL-4 reporter mice (*Il13r2*) using Biologend mAb, huCD2-biotin (5T1/8). Th2 cells were identified as IL-4–expressing CD4⁺, stained with eBioscience mAb, CD4-efluor V450 (Ly-2), IL-13 expression was determined using IL-13eGFP reporter mice (6). IL-5 expression was determined by intracellular staining. In brief, cells were fixed and permeabilized using the BD CytoFix/Cytoperm kit (BD Biosciences) and stained with BD Biosciences mAbs, IL-5–PE (TRFK5). To identify eosinophils and AAM, we stained cells with BD Biosciences mAbs (Siglec-F–PE [E50-2440], F4/80–FITC mAb [DJ8: MD Biosciences]). To identify ILC2, type I and type II NKT cells, and in vitro–generated Th2 cells, we stained cells with BD Biosciences mAbs (CD8–allophycocyanin [Ly-2], Gr-1, FcγR1-allophycocyanin [MAR-1], and T1/ST2–FITC mAb [DJ8: MD Biosciences]). To identify eosinophils and AAM, we stained cells with BD Biosciences mAbs (Siglec-F–PE [E50-2440], F4/80–allophycocyanin [BM8] and eBioscience mAb (CD11b-PerCP [M1/70]). IL-4 expression in the cells was identified in IL-4 reporter mice (*Il13*). NKT cells were gated as described earlier and sorted on a MoFlo cell sorter. The purity of the sorted ILC2 was 97%. C57BL/6J mice were treated with 2 µg recombinant mouse IL-25 i.p. for 3 d, and the peritoneal exudate cells were collected and stained for ILC2 isolation. ILC2 were gated as described earlier and sorted on a MoFlo cell sorter. The purity of the sorted ILC2 was >97%.

C57BL/6J mice were treated with α-Gal-Cer or sulfatides i.p., and spleen cells were recovered and stained for type I and type II NKT cells, as described earlier, and sorted on a MoFlo cell sorter. The purity of the sorted populations revealed a purity >97%.

For ILC2, type I and type II NKT cells, and in vitro–generated Th2 cells (9), 200,000 cells were transferred i.p. on days 0 and 2. Organs were collected on day 6.

**Statistics**

Statistical analysis was performed using GraphPad InStat. Results are presented as mean ± SEM. Differences, indicated as two-tailed *p*-values, were considered significant when *p* < 0.05, as assessed by unpaired Student *t* test with Welch correction applied as necessary.

**Results and Discussion**

The absence of IL-13 is associated with weight gain because of significantly reduced eosinophils and AAM in VAT

The CAM versus AAM polarization state of macrophages in the VAT governs local insulin sensitivity and prevents the development of metabolic syndrome and type 2 diabetes (10). Recently, Wu et al. (3) demonstrated that IL-4 release from eosinophils, induced by IL-5, could induce weight loss in obese mice, through sustaining AAM polarization in VAT. In addition, another of the type 2 cytokines, IL-13, has been identified as particularly important in glucose homeostasis, a dysregulation of which is implicated in the generation of type 2 diabetes and weight gain (5, 11). Indeed, mice deficient in IL-13 (*Il13*⁻/⁻) fed an HFD have markedly exacerbated weight gain when compared with comparable treated wild-type (WT) animals (Fig. 1A) (11), with *Il13*⁻/⁻ mice demonstrating dysregulated glucose metabolism and glucose intolerance when compared with WT counterparts (Fig. 1B) (11). We analyzed the cellular composition of the VAT in *Il13*⁻/⁻ mice on HFD and observed a significant (*p* < 0.05) decrease in both eosinophils and AAM compared with WT mice (Supplemental Fig. 1A), which would contribute to the glucose intolerance and exacerbated weight gain in *Il13*⁻/⁻ animals. In addition to the observed localized effects on glucose metabolism, a key role for IL-13 in hepatic glucose production suggests that IL-13 exhibits an effect beyond that observed in modulating inflammation (11).

IL-13 is produced by a number of immune cells that can be found in VAT, such as Th2 cells; however, work has recently focused on ILC2 (4, 6) as innate cellular sources of IL-13. Indeed, a role for ILC2 in regulating weight gain through the release of IL-5 and IL-13, thereby sustaining eosinophils and AAM in VAT, was recently described (5). Because of the clear importance of IL-13 in regulating weight gain, we used dual IL-4/IL-13 reporter mice (*Il13kn2*/+/Il13eGFP) (6, 8), to determine the predominant cellular sources of IL-13 and also IL-4 in VAT in obese mice. In adipose tissue, lineage marker (CD3, CD4, CD8, CD11b, CD11c, CD19, F4/80, Gr-1, FcεR1)–negative innate cells are the major producers of IL-13 (Fig. 1C). In VAT, ILC2 (Lineage⁺ ICOS⁺/T1/ST2⁺IL-7Rα⁻ and NKT (TCRB⁺/NK1.1⁺) subpopulations are the prominent IL-13–producing cells (Fig. 1D). NKT cells are present in VAT of both mice and humans (12), and are defined as classical type I invariant NKT cells, which specifically express the receptor Vα14/Vα18 in mice, or nonclassical

*FIGURE 1.** IL-13 produced by type 2 cells regulates weight gain and glucose tolerance in mice. (A) Weight gain, expressed as a percentage from starting weight, in *Il13*⁻/⁻ and WT mice on HFD for 8 wk. (B) Blood glucose tolerance test. (C) Percentage of IL-13⁺ eosinophils, AAM, Th2 cells or innate cells, and (D) IL-13–expressing innate cells in VAT. (E) IL-4 and/or IL-13 expressing ILC2, type I or type II NKT cells in *Il13kn2*/+/Il13eGFP mice. Data are representative of *n* = 2–6 (± SEM) from two independent experimental replicates (*p* < 0.05, **p** < 0.01).
type II NKT cells with a variable TCR repertoire (13). We show that although type II NKT cells are the predominant source of IL-13, type I NKT cells predominately expressed IL-4 in the VAT, whereas eosinophils are the major source of IL-5 (Fig. 1D, 1E; data not shown). Some recent studies have demonstrated that type I NKT cells are depleted in VAT of obese individuals (12, 14). Furthermore, mice deficient in both populations of NKT cells (Cd1d<sup>−/−</sup>) and specifically type I NKT cells (J<sub>o18<sup>−/−</sup></sub>) have shown increased weight gain and elevated fasting glucose, whereas transferring type I NKT cells back into J<sub>o18<sup>−/−</sup></sub>-deficient mice improves glucose homeostasis (12, 14). However, other studies have shown no role for NKT cells in either weight loss or glucose homeostasis (15). The role for type II NKT cells is less defined, although a recent study suggests that type II NKT cells initiate inflammation in the adipose tissue, which may result in insulin resistance (16). A variety of factors are postulated to explain discrepancies between studies on NKT cells and obesity in mice (17). In the context of this study, male C57BL6/J congenic Cd1d<sup>−/−</sup> or J<sub>o18<sup>−/−</sup></sub> mice have no significant alteration in either diet-induced obesity or glucose homeostasis when compared with WT mice maintained in our animal facility (data not shown).

**IL-25 induces weight loss and IL-13–producing cells in obese mice**

As outlined earlier using the Il4<sup>knockout</sup>/Il13<sup>egfp</sup> mice, ILC2 and type II NKT cells are prominent cellular sources of IL-13 in the VAT from both obese and lean mice (Fig. 1D, 1E; data not shown). Because the type 2 cytokine IL-25 drives expansion of ILC2 (6, 18), we treated obese mice with IL-25 and monitored metabolic and inflammatory changes. In addition, mice were also treated with the glycolipid ligand α-Gal-Cer to induce type I NKT cells (19) or sulfatides (3′-sulfated b-galactosylceramide) to induce type II NKT cells (20). Exogenous treatment of obese mice with each reagent preferentially expanded the frequency of respective cell types in VAT (Fig. 2A–C).

Administration of α-Gal-Cer specifically induces type I NKT cells, whereas administration of sulfatides induced type II NKT cells and also ILC2 (Fig. 2A–C). Although all treatments significantly (<p>0.001) induced eosinophilia in the fat, only treatment with IL-25 significantly (<p>0.05) induced AAM (Supplemental Fig. 1B). The ability of IL-25 and sulfatides to induce both ILC2 and type II NKT cells suggests an intricate interplay between these cell types. Indeed, a role for IL-25 in inducing both IL-13–producing ILC2 and IL-17R<sup>B</sup> NKT cells has been reported in mouse models of airways hyperreactivity and colitis (21, 22). Interestingly, although we show that both type I and type II NKT cells express IL-17BR, only type II NKT cells appear to be induced by IL-25 treatment (Fig. 2B, 2C, Supplemental Fig. 2).

In addition to the clear effects of governing cellularity of the VAT, we show that inducing either type I or type II NKT cells in obese mice induces significant (<p>0.05) weight loss (Fig. 2C), with a significant (<p>0.01) reduction in fasting glucose, almost returning levels to those seen in mice fed normal chow (data not shown), and improved glucose tolerance (Fig. 2D), suggesting improved glucose homeostasis in response to expansion of type I or type II NKT cells. This weight loss in obese mice only is associated with significant (<p>0.05–0.001) reduction in the size of both visceral and s.c. adipose tissue (Fig. 2F, Supplemental Fig. 1C), and in the case of sulfatides is not associated with a corresponding reduction in food or water intake, although food and water intake do appear slightly reduced in mice treated with α-Gal-Cer (Supplemental Fig. 3D, 2017).

Because of the ability of IL-25 to drive the expansion of ILC2 and NKT cells (Fig. 2A), both of which are able to drive weight loss (5, 12), we assessed the ability of IL-25 to induce weight loss. Treating obese mice with rIL-25 induced significant (<p>0.05) weight loss and glucose intolerance (Fig. 2E, 2F). Treatment with IL-25, although resulting in significantly decreased VAT and SAT (Fig. 2F, Supplemental Fig. 1C), does not alter food or water intake (Supplemental Fig. 1D), suggesting that the weight loss observed is due to the altered cellularity of the adipose tissue. It is also noteworthy that treatment with IL-25 does not alter weight gain in mice fed a control diet (Supplemental Fig. 1E).

**Depletion of ILC exacerbates weight gain in mice**

We have identified a role for IL-25–induced IL-13–producing ILC in regulating the cell composition of VAT and inducing weight loss. To confirm a role for ILC2 specifically...
in metabolic homeostasis, we used T and B cell–deficient \textit{Rag1}\textsuperscript{−/−} mice and treated the mice with anti-CD90.2 mAb to block ILC2 (23). It is noteworthy that \textit{Rag1}\textsuperscript{−/−} mice are capable of gaining weight in response to HFD (Fig. 3A), indicating that weight gain is independent of adaptive immunity. In \textit{Rag1}\textsuperscript{−/−} mice treated with anti-CD90.2 mAb there was significantly (\( p < 0.05 \)) greater weight after HFD treatment relative to control mAb-isotype–treated \textit{Rag1}\textsuperscript{−/−} mice with reduced glucose tolerance (Fig. 3A, 3B). This was apparent not only in total body weight, but anti-CD90.2–treated mice also had significantly (\( p < 0.05 \)) increased s.c. and VAT deposits (Fig. 3C–E). Interestingly, anti-CD90.2 treatment also significantly (\( p < 0.05 \)) enhanced weight gain in mice fed a control diet (Supplemental Fig. 2A). However, treatment of either CD or HFD fed mice did not alter food or water intake (Supplemental Fig. 2B, 2C). Anti-CD90.2 mAb–treated mice had significant reductions in ILC2 (\( p < 0.01 \); Fig. 3C), with attendant reductions in eosinophils and AAM in VAT of \textit{Rag1}\textsuperscript{−/−} mice treated with anti-CD90.2 (Supplemental Fig. 2D), suggesting the deletion of ILC2 alone can affect eosinophil and subsequent AAM polarization independently of NKT cells, which are absent in \textit{Rag1}\textsuperscript{−/−} mice. This provides further evidence that in this mouse model of obesity, ILC2 play an important role in regulating weight gain by promoting localized eosinophilia and AAM polarization. However, these studies in \textit{Rag1}\textsuperscript{−/−} mice do not allow for the interaction of T or B cells with other cells, which may be of significant importance in normal animals.

To elucidate the relative roles for ILC2 and NKT cell types in diet-induced obesity and glucose homeostasis, we transferred ILC2, type I or type II NKT cells into obese mice. In accordance with previously published data (12), transfer of type I NKT cells induces a transient weight loss and improves glucose tolerance (Fig. 4). Transfer of ILC2 caused a transient weight loss and improvement in glucose tolerance comparable with that observed in mice receiving type I NKT cells (Fig. 4A, 4B, Supplemental Fig. 2E). Of particular interest is the effect of transferring type II NKT cells, which induces a greater and more prolonged weight loss and marked glucose tolerance (Fig. 4A, 4B, Supplemental Fig. 2E). Transfer of ILC2, type I or type II NKT cells induced eosinophilia and AAM polarization in the VAT relative to control mice, with transfer of ILC2 predominantly inducing AAMs, whereas type I and type II NKT cells induce high levels of both eosinophils and AAMs (Supplemental Fig. 2F). It is noteworthy that the transfer of in vitro expanded Th2 cells does not induce significant weight loss or alter glucose tolerance. These data show that although all three innate cell types play an important role in regulating weight gain and glucose homeostasis, there appears to be a hierarchy of action, with type II NKT cells potentially, on a cell transfer basis, having a more important role.

The interaction between the innate immune system and obesity is a prominent area of research because of the current health crisis associated with the obesity epidemic. In addition to providing mechanistic insights, this study suggests a future potential role for modulating cytokine activity in the adipose tissue of obese patients, either to upregulate IL-25 or IL-13 directly, thereby artificially promoting ILC2 and NKT cell expansion and localized eosinophilia and AAM polarization. It would also be prudent to assess the effects of upregulating these cytokines on other metabolic organs. This study raises the potential for IL-25 and IL-25 elicited cells as therapeutics for stabilizing glucose homeostasis and weight.

\textbf{Acknowledgments}  
We thank Alan Nolan for technical assistance. Prof. Masaru Taniguchi (RIKEN Research Center for Allergy and Immunology, Kanagawa, Japan) kindly provided Jo18\textsuperscript{−/−} mice. We are grateful to the National Institutes of Health Tetramer Core Facility for providing CD1d tetramers.
Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure 1

A. Eosinophils
- WT
- Il13<sup>−/−</sup>

B. Eosinophils
- PBS
- α-Gal-Cer
- IL-25
- Sulfatides

C. Mice
- VAT
- SAT

D. Food intake (g)
- PBS
- α-Gal-Cer
- IL-25
- Sulfatides

E. Weight change (%)
- PBS
- IL-25
- α-Gal-Cer
- Sulfatides

F. Weight change (%)
- WT
- Il17br<sup>−/−</sup>

G. Glucose (mM/L)
- WT
- Il17br<sup>−/−</sup>
Supplemental Figure 2

A. Weight gain (%) over time (weeks).

B. Food intake (g) between CD and HFD groups.

C. Water intake (ml) between CD and HFD groups.

D. Flow cytometry images showing changes in eosinophils and AAM with IgG and anti-CD90.2 treatment.

E. VAT weight (g) over time (weeks).

F. Cells per g of VAT for different cell types with significance levels indicated.
IL-25 elicits innate lymphoid type 2 and type II natural killer T cells that regulate obesity in mice

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Supplemental Figure Legends

Supplemental Figure 1: Supporting data for IL-25, α-Gal-Cer and sulfatide treatment.

Eosinophils (CD11B⁺SiglecF⁺) and AAM (CD11b⁺F4/80hi) infiltration in the VAT of obese C57BL6/J (WT) and Il13⁻/⁻ mice (A). Groups of C57BL6/J mice were treated with PBS, IL-25, α-Gal-Cer or sulfatides on days 0, 2 and 4 with analysis of VAT on day 6. (B) eosinophils (CD11B⁺SiglecF⁺) and AAM (CD11b⁺F4/80hi) infiltration in the VAT. (C) Photographs of obese mice and excised VAT and SAT [L-R: PBS treated, IL-25 treated, α-Gal-Cer treated, sulfatide treated]. (D) Food and water intake expressed per day, per mouse. (E) weight change of lean mice treated with PBS, IL-25, α-Gal-Cer or sulfatides on days 0, 2 and 4. WT and Il17br⁻/⁻ mice were fed HFD and (F) their weight monitored over 8 weeks and expressed as the percentage weight gain from the starting weight. (G) Glucose tolerance was assessed in both groups in response to injection of 2 g/kg glucose. Data are representative of n=3-6 (+/- SEM) from 3 independent experimental replicates (ns - not-significant, *P<0.05, ***P<0.001).

Supplemental Figure 2: Supporting data for Rag-1⁻/⁻ anti-CD90.2 treatment and adoptive transfer experiments.

Rag-1⁻/⁻ mice were treated with control IgG or anti-CD90.2 mAb every 3 days while on a HFD for 8 weeks. (A) Weight gain in mice fed a control diet and treated with control IgG or anti-CD90.2. Food (B) and water (C) intake for mice fed either control (CD) or high-fat
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(HFD) diet expressed per mouse per day. (D) Eosinophil and AAM induction in the VAT. In groups of obese C57Bl6/J 200,000 Th2 cells, ILC2, type I NKT or type II NKT cells were transferred i.p. on day 0 and day 2 and VAT analyzed on day 6. (E) VAT weight and (F) VAT eosinophil and AAM infiltration. Data are representative of n=5-6 (+/- SEM) from 3 independent experimental replicates (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).