FULL PAPER *Immunology*

Regulation of type 2 diabetes by helminth-induced Th2 immune response

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ABSTRACT. Helminth-induced type 2 cytokines increase the number of regulatory T cells and alternatively activated macrophages, resulting in modulation of the host–immune system. Studies on these parasite-induced immunoregulatory mechanisms might contribute to the development of new therapies for inflammatory diseases, including type 2 diabetes (T2D). Previous studies have suggested that progression of obesity-associated metabolic abnormalities is under pathophysiological control of CD4+ T cells. Glucose absorption through the intestinal epithelium reduced after infection in a STAT-6-dependent manner. In this study, we investigated whether infection with the gastrointestinal nematode parasite *Heligmosomoides polygyrus* (Hp) can modulate T2D-associated pathology in a mouse model (KK-Ay/TaJcl). KK-Ay/TaJcl mice were inoculated with infective third-stage Hp larvae and studied at Day 8 following infection. Uninfected KK-Ay/TaJcl mice showed high blood glucose levels even 120 min after administration of glucose by IP injection. However, it was significantly improved in the infected group. HOMA-IR, fat accumulation and FAS gene expression in the liver were significantly decreased by Hp infection. GLUT2 gene expression in this group was significantly lower than that in the uninfected diabetic mice, which might be related to the decrease in glucose absorption in the parasite-infected intestine. In conclusion, helminth-induced type 2 immune responses might contribute to T2D disease control.

KEY WORDS: alternatively activated macrophages, GLUT2, steatosis, type 2 cytokines, type 2 diabetes

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Inappropriate immune responses, including cytokine imbalances, contribute to the development of persistent diseases [32]. Excessive activation of the immune system may cause chronic inflammation, which is central to the pathogenesis of several diseases [2]. Type 2 diabetes (T2D) is a chronic inflammatory disease characterized by persistent elevated glucose levels due to insulin resistance. Insulin resistance is a condition in which the body is unable to use insulin appropriately. Overeating or physical inactivity may lead to obesity, including infiltration of adipose tissue by macrophages. Macrophages are initially activated by the classical pathway, but keep producing inflammatory cytokines. Macrophage-induced tumor necrosis factor α (TNFα) promotes insulin resistance by inhibitory serine phosphorylation of insulin receptor substrate 1 (IRS 1) [13, 38].

Treatment of T2D is based on proper diet and exercise, with a view to maintain appropriate weight, control blood glucose levels and improve insulin tolerance. There are several medications available for T2D, including insulin sensitizers, secretagogues (which stimulate insulin release), alpha-glucosidase inhibitors (which retard the digestive/absorptive process), dipeptidyl peptidase-4 (DPP-4) inhibitors (which inhibit DPP-4—the enzyme that inactivates incretin hormones, GLP-1 and GIP), incretin mimetics and sodium–glucose transporter (SGLT2) inhibitors. However, there is no definite drug treatment for everyone, and complete cure is rarely seen. Patients must try to change their life styles and collaborate with doctors to determine specific medication to prevent exacerbation of their condition. Some studies have sought to improve insulin resistance through immunotherapy [3, 6, 14, 16, 40].

Helminth infection induces strong host Th2 responses. IL-4 and IL-13 are cytokines important for alteration of the physiological function of the small intestine, including the expelling of worms [48]. These cytokines induce alternatively activated macrophages (AAMacs or M2 macrophages), which downregulate inflammatory responses [1]. Previous research reported that helminth infection or immunization with eggs is protective in murine models of asthma [17], multiple sclerosis [41], type 1 diabetes (T1D) [4, 20] and inflammatory bowel disease [42]. Helminth-induced type 2 cytokines increase regulatory T cells (Treg) [47] and AAMacs [1], resulting in inhibition of the production of excess inflammatory cytokines, so as to modulate the immune system of the host. Studies of these parasite-induced immunoregulatory mechanisms might contribute to developing new therapies for inflammatory diseases, including T2D. However, the mechanism underlying the immunomodulation-based effect is still unclear. Winer *et al*. focused on CD4+ T cells resident in adipose tissue controlling insulin resistance in a mouse model. Their results suggested that progression of obesity-associated metabolic abnormalities is under pathophysiological control of CD4+ T cells [49]. Meanwhile, Madden *et al*. showed reduced glucose absorption through the intestinal epithelium following nematode infection [23], and although the mechanism underlying this phenomenon is still unclear, it appears to be STAT-6 dependent, suggesting that

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the decrease in glucose absorption on nematode infection is associated with increase in type 2 cytokines. Nevertheless, treatment of T2D has several potential target points, and inhibition of glucose absorption in the small intestine is one of the effective treatments being used to avoid rapid postprandial blood sugar elevation. Therefore, alpha-glucosidase inhibitors are effective, as mentioned above. For this reason, understanding the molecular mechanism underlying inhibition of glucose absorption following nematode infection might contribute to developing new therapeutic strategies. Of particular interest is whether type 2 cytokines may contribute to glucose homeostasis.

In terms of glucose homeostasis, a diet rich in monounsaturated fatty acids (MUFAs) improves blood lipid levels and glucose metabolism [7–9, 15, 37]. Dietary MUFA involves the insulin signaling pathway and expression of glucose transporters in muscle tissue [26]. MUFA activate peroxisome proliferator-activated receptor δ (PPARδ) to enhance tissue AAMacs maturation [36]. PPARs are transcription factors belonging to the nuclear receptor superfamily, and the PPAR family consists of three subtypes—PPARα, PPARγ and PPARδ—all of which are regulated by fatty acids and their derivatives; they modulate lipid and glucose homeostasis. Macrophage-specific PPARγ or PPARδ knockout mice developed insulin resistance, including a reduced number and impaired function of AAMacs [35, 36]. Therefore, we hypothesized that nematode-induced type 2 cytokines could have important roles in controlling diabetes in mice.

In this study, we investigated whether nematode infection can modulate T2D pathology through cytokine regulation in a T2D mouse model. In addition, we were interested in the alteration of glucose transporters in the small intestine on nematode infection. Our results might contribute to developing immunotherapy for T2D.

MATERIALS AND METHODS

Mice: Male KK-Ay/TaJcl mice (6 weeks old) were purchased from CLEA Japan, Inc. (Tokyo, Japan) and used for all experiments. The animals were housed under conventional conditions, given food and water *ad libitum*, and mainained in a 12-hr/12-hr light/dark cycle. The environment was maintained at 22 ± 1.5°C, with a relative humidity of 55 ± 5%. The mice were divided into two groups, and body weight and plasma glucose levels were measured once a week. The experimental protocol was approved by the Institutional Ethics Commission for Animal Research of Miyagi University.

Parasites: *Heligmosomoides polygyrus* (Hp) was provided by Dr. Kenji Ishiwata (The Jikei University School of Medicine (Tokyo, Japan)) and maintained in ICR mice. Eleven-week-old KK-Ay/TaJcl mice were orally inoculated with 200 L3 larvae of Hp. Control mice received saline orally during the same period. Mice were sacrificed on Day 8 after the inoculation, and the small intestine, liver, fat tissues and blood were collected.

Gene expression analysis: *RNA extraction, reverse transcription and real-time PCR*: Total RNA extraction from whole tissue was performed using TRIzol Reagent (Life Technologies, Inc., Frederic, MD, U.S.A.) according to the manufacturer's instructions. RNA levels were measured on a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, U.S.A.), and cDNA was synthesized with random primers and SuperScript II (Life Technologies, Inc.). Primer sequences for IL-4, IL-13 [30], IL-10 [10], Arginase 1 (ARG1), FIZZ1, YM1 [54], fatty acid synthase (FAS) [52] and GLUT2 [34] have been described previously. Real-time PCR was performed using Brilliant SYBR Green QPCR Master Mix III (Stratagene, La Jolla, CA, U.S.A.) with an MX3000P system (Stratagene). Amplification conditions were as follows: 95°C for 3 min, 40–50 cycles of 95°C for 5 sec and 60°C for 20 sec. Fluorescence signals measured during the amplification were analyzed. Ribosomal RNA primers were used as an internal control, and all data were normalized to constitutive rRNA values. Quantitative differences between the groups were calculated according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, U.S.A.).

Laser capture microdissection (LCM): LCM was performed as described in previous reports [28, 29]. A cryosectioned tissue was stained with H&E and dehydrated, and LCM was performed using PicCell II (Arcturus Engineering, Mountain View, CA, U.S.A.). Cells were obtained from the epithelial region of the small intestine and transferred to CapSure LCM Caps (Arcturus Engineering). Total RNA was extracted using an RNA isolation kit (Qiagen, Redwood city, CA, U.S.A.), and cDNA was synthesized and real-time PCR was performed as described above.

Preparation of frozen and paraffin blocks and sectioning: Small pieces of the small intestine were slit longitudinally, laid flat with the mucosal surface facing down, rolled around a wood stick (Swiss roll) and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek U.S.A., Inc., Torrance, CA, U.S.A.) in a cryomold. A part of the liver was also embedded in Tissue-Tek O.C.T. Compound. The tissue samples were frozen using dry ice–acetone, then removed from the cryomold and stored at −80°C in an airtight container until sectioning. Four-micrometer-thick tissue sections were obtained from the frozen blocks using plain coated slides and an HM560 cryostat (Carl Zeiss, Oberkochen, Germany). The slides were immediately placed on dry ice and stored at −80°C until analysis. The frozen slides of the small intestine tissues were used for LCM, and liver tissues were used for Oil-O-Red staining. At the same time, paraffin blocks of liver and small intestine tissues were fixed using 10% formaldehyde and embedded in paraffin for pathomorphological examination using H&E staining.

Immunofluorescence staining: *Macrophages*: Four-micrometer-thick frozen tissue sections were fixed in cold acetone for 20 min and incubated in 10% normal rat serum and 1 μg/m*l* of an affinity-purified rat anti-mouse CD16/CD32 antibody (BD Biosciences, San Jose, CA, U.S.A.) in PBS for 20 min at room temperature. After washing with PBS, the tissue samples were incubated with 10 μg/m*l* of an FITC-conjugated rat anti-mouse CD206 antibody (BioLegend Inc., San Diego, CA, U.S.A.) and 25 μg/m*l*

of an Alexa647-conjugated rat anti-mouse F4/80 antibody (BioLegend Inc.) in tandem in PBS containing 0.1% BSA overnight at 4°C. Next, the tissue samples were washed in PBS, coverslipped with Vectorshield (Vector Laboratories, Burlingame, CA, U.S.A.), examined and digitally photographed using an Axio Imager microscope with Axio Vision 4.6 software (Carl Zeiss, Oberkochen, Germany). *GLUT2*: Four-micrometer-thick tissue sections were fixed in cold acetone for 20 min and incubated in 10% normal goat serum in PBS for 1 hr at room temperature. The tissue sections were then exposed to anti-GLUT2 antibody (1:1,000 dilution) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.), which was diluted in PBS containing 10% normal goat serum, 0.3% Triton X and 0.1% sodium azide, overnight at 4°C. After incubation, the sections were washed in PBS and incubated with a secondary antibody (Alexa Fluor 488, goat anti-rabbit; Molecular Probes, Eugene, OR, U.S.A.), which was diluted 1:200 in PBS containing 10% normal goat serum, 0.3% Triton X and 0.1% sodium azide for 2 hr at room temperature. The rest of the procedure was similar to that of the macrophage staining described above.

Intraperitoneal glucose tolerance test (IPGTT): At Day 8 after infection, both groups of mice were peritoneally injected with 2 g/kg body weight glucose after 16 hr (overnight) of fasting, and whole blood was collected at 0, 15, 30, 45, 60, 90 and 120 min on injection. Blood glucose was measured using the Fuji Dri-Chem system (FUJIFILM Corp., Tokyo, Japan). Serum insulin concentration was measured with a mouse insulin ELISA kit (Shibayagi Co., Ltd., Shibukawa, Japan) for HOMA-IR. HOMA-IR is a method used to calculate insulin resistance [25] according to the following formula: fasting insulin (μU/*l*) ×fasting glucose (nmol/*l*)/22.5.

Liver enzyme test: Blood samples were obtained from the mice at Day 8 after infection, and glutamic–pyruvic transaminase/alanine aminotransferase (GPT/ALT) was measured using the Fuji Dri-Chem system for analysis of liver function.

Extraction and measurement of total lipids: Total lipids in the liver were extracted and analyzed according to the Folch method [5]. Briefly, frozen liver tissue (1 g) was homogenized in 10 m*l* of chloroform:methanol (2:1) and the homogenate was filtrated. The filtrate was mixed with 40 m*l* of saturated saline solution. After leaving the mixture for more than 10 min, the upper phase was removed by aspiration. The lower chloroform phase containing lipids was dehydrated with sodium sulfate and evaporated under vacuum in a rotary evaporator, and total lipid weight was determined by reweighing the tube.

Statistical analysis: All data were calculated as means ± SE for each treatment group. Differences in mRNA expression among the groups were determined using the *t*-test. A difference with a probability (*P*) value <0.05 was considered statistically significant. Appropriate time- and age-matched controls were used for each group (*N*=3–5 controls for each group).

RESULTS

Blood glucose levels before and after infection: KK-Ay/TaJcl mice are well known as a T2D model that develops obesity and hyperglycemia at early stages [33]. This strain was obtained by introducing the Ay gene into the KK background and developed a relatively higher blood glucose level compared with other strains. Figure 1A shows the changes in body weight of the Hp-infected and uninfected KK-Ay/TaJcl mice throughout the experimental period. The body weight increased in both groups at 6–12 weeks (Fig. 1A), and the mice were orally inoculated with Hp or saline at 11 weeks. All mice showed high blood glucose levels (more than 400 mg/d*l*) at the time of infection (Fig. 1B).

To examine whether physiological changes in nematode-infected mice contribute to improving the diabetic condition, the mice were examined at Day 8 on infection. Figure 1C shows the results of the intraperitoneal glucose tolerance test for both groups. The KK-Ay/TaJcl mice showed high glucose levels even 120 min after administration of glucose by IP injection; however, levels had significantly improved in the infected group. HOMA-IR, which has been commonly used as an insulin resistance index, was also significantly reduced in the infected group (Fig. 1D).

Fat accumulation in the liver: The mechanism involved in T2D-associated fatty liver is unclear, but hepatic steatosis is frequently observed in T2D patients. Figure 2 shows representative livers of the KK-Ay/TaJcl mice. Diabetic mice have fatty livers, which can be confirmed by the naked eye and by pathological studies (Fig. 2A–2C). After Hp infection, fat accumulation in the liver was still observed, but had markedly declined (Fig. 2D–2F). The gene expression of FAS, which is an important gene for lipogenesis, significantly decreased in the Hp-infected group (Fig. 2G). GPT, also known as ALT, is a biomarker that indicates the liver health. Patients with fatty liver tend to show a higher level of GPT. However, GPT serum levels were significantly lower in the Hp-infected group (Fig. 2H). Furtheremore, fat content in the liver tended to be lower in the infected group (Fig. 2I).

Cytokine gene expression and macrophage activation in the small intestine: H&E staining revealed that inoculated larvae moved into the small intestine and got embedded in the submucosa by Day 8 after infection (data not shown). Previous studies have shown that expression of type 2 cytokines is markedly elevated at Day 8 after Hp infection in BALB/c mice [31]. However, the peak was lower in C3H/HeN and C57BL/6J mice than in BALB/c mice. In this study, we investigated whether gene expression of type 2 cytokines in KK-Ay/TaJcl mice was upregulated after Hp infection. The gene expression of type 2 cytokines in the intestine had significantly and prominently increased compared with that in the uninfected diabetic mice (Fig. 3A–3C). In addition, ARG-1, FIZZ1 and YM1, which are markers of AAMacs, were also markedly upregulated in the infected group (Fig. 3D–3F), indicating that the AAMacs were induced and accumulated in the intestine on Hp infection. To confirm the results of gene expression, immunofluorescence staining was performed. A large number of CD206+/F4/80+ cells, which

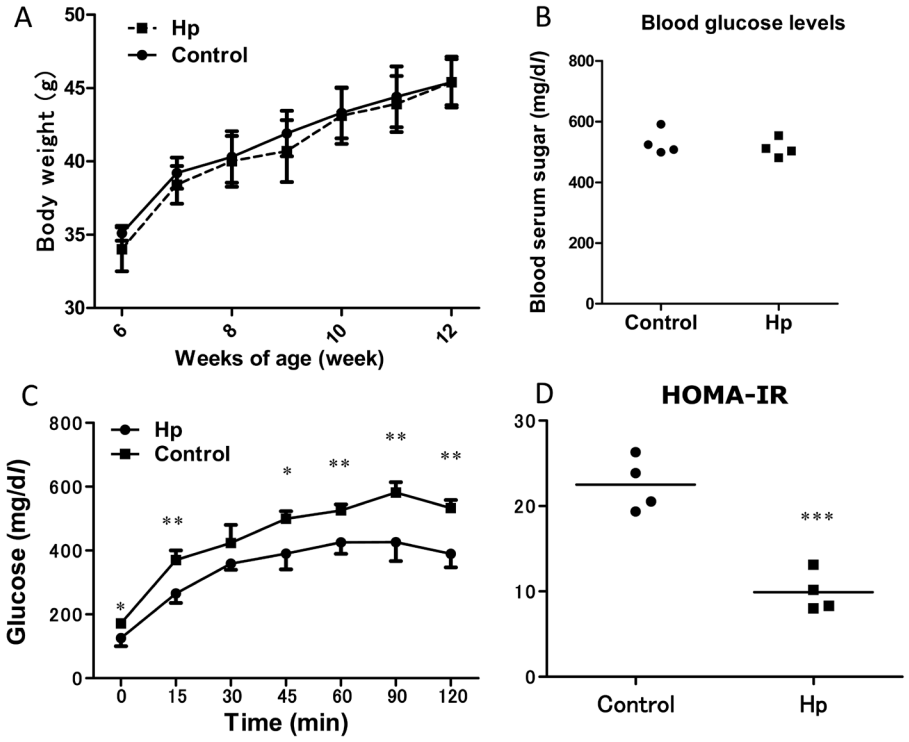

Fig. 1. The KK-Ay/TaJcl mouse strain develops obesity and insulin resistance at early stages. (A) The KKAy/TaJcl mice increased their body weight. The mice were divided into two groups at 6 weeks of age, and one group was infected with Hp and investigated on day 8 after infection. (B) Blood glucose levels at the time of infection. (C) IPGTT was significantly decreased in the Hp-infected mice. (D) Insulin resistance index (HOMA-IR) was recovered in the Hp-infected KK-Ay/TaJcl mice. **P*<0.05; ***P*<0.01; ****P*<0.001. *N*=4. We performed two independent experiments with similar results.

are the cell surface markers of AAMacs, were observed in the Hp-infected group (Fig. 3G). It is well known that AAMacs are induced by IL-4 and IL-13. Wu *et al*. showed clearly that eosinophils play an important role in alternative macrophage activation [51]. Our pathomorphological examination confirmed that many eosinophils accumulated in the submucosa of the intestine of Hp-infected diabetic mice intestine (Fig. 4). Upregulated IL-10 and activated AAMacs could be responsible—at least in part—for improving inflammatory status.

Glucose transporters in the small intestine: A previous study revealed that glucose absorption in the small intestine is inhibited in nematode-infected mice [23]. This phenomenon was not observed in STAT-6 KO mice, suggesting that type 2 cytokines alter some physiological conditions involving glucose absorption in the small intestine. We hypothesized that glucose transporters (GLUTs) in the epithelial membrane might be subject to change in number or location on nematode infection. GLUTs are responsible for the passive transport of glucose across the cell membrane [50]. SGLT1, which mediates the uptake of both glucose and galactose, is the sodium-dependent transporter at the brush-border membrane (BBM). GLUT2 is mainly expressed in the basolateral membrane of enterocytes to transport glucose into the blood vessels, but is recruited to the BBM by a simple sugar meal [12]. Development of diabetes is associated with the elevation of GLUT expression, and GLUTs are considered to be therapeutic targets in diabetes. Figure 3 shows elevated IL-4 and IL-13 gene expression at Day 8 after Hp infection. At the same time, although the SGLT1 expression showed no change after Hp Infection (Fig. 5A), the GLUT2 expression in the small intestine of the Hp-infected mice was significantly lower than that in the intestine of uninfected diabetic mice (Fig. 5B). Next, the intestinal epithelial cells were dissected using LCM and analyzed. In the Hp-infected group, epithelial gene expression of GLUT2 was about half of that observed in the uninfected mice (Fig. 5C). To confirm the results of the gene expression, immunofluorescence staining was performed, and it revealed a weaker positive staining of GLUT2 in the small intestine of the infected mice compared with that in the uninfected mice (Fig. 5D).

DISCUSSION

It is well known that imbalances in cytokine levels result in autoimmune diseases, including chronic inflammatory diseases [2, 32, 46]. Several previous reports have demonstrated the therapeutic potential of parasite infection for curing immune dysfunctions [19, 24, 44, 45]. One of the mechanisms behind this beneficial effect is thought to

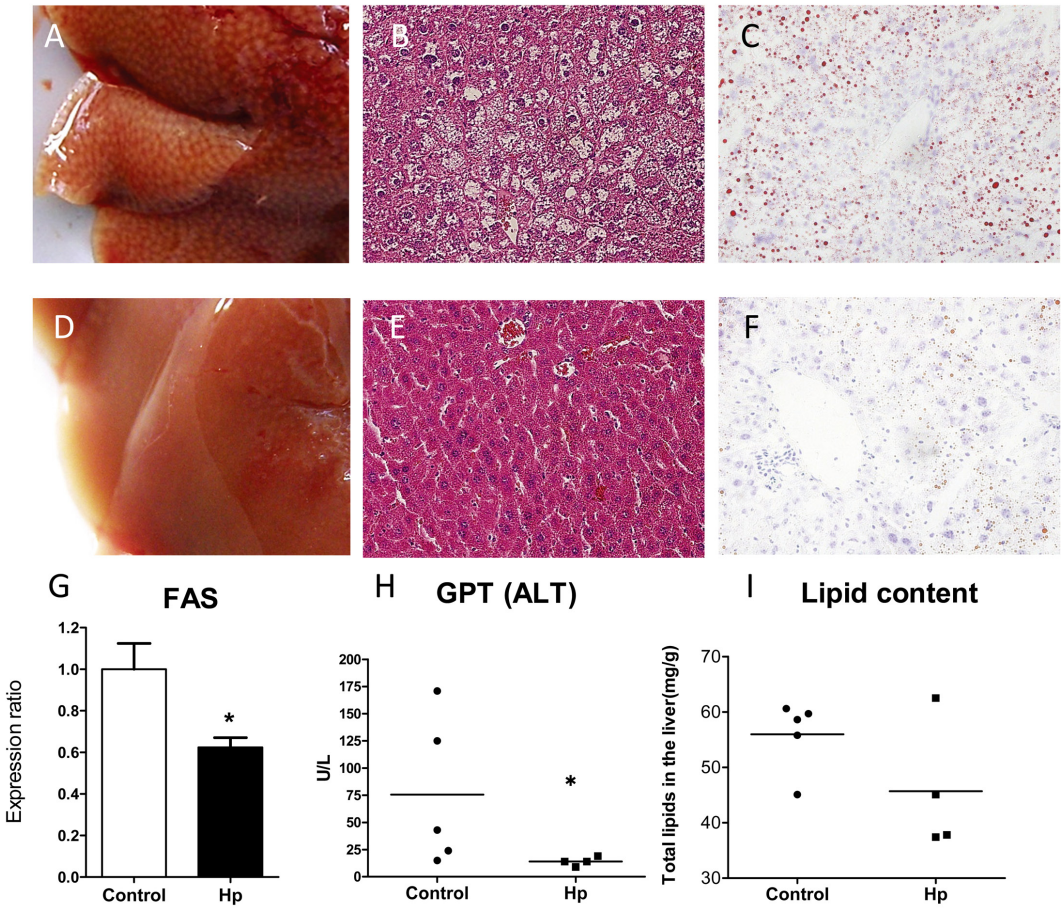

Fig. 2. Representative liver from uninfected (A) and Hp-infected (D) mice (12 weeks old, day 8 after infection). H&E-stained histology (B: uninfected, E: infected) and Oil-O-Red staining (C: uninfected, F: infected) for assessment of fat accumulation. All images of BCEF were acquired at 200× magnification, and the images are representative for more than four mice. FAS gene expression in the liver (G), GPT (ALT) in serum (H) and total lipid content in the liver (I). *P<0.05, N≥4.

be due to nematode-induced Th2 immune responses moderating immunopathological responses toward excessive inflammatory cytokines. Our study suggested that parasite-induced Th2 immune responses prevented type 2 diabetes in KK-Ay/TaJcl mice. IPGGT was significantly improved on nematode infection (Fig. 1C). HOMA-IR was also markedly decreased (Fig. 1D), suggesting that insulin sensitivity was recovered on Hp infection. Because the elevation of type 2 cytokines induces AAMacs, which inhibit inflammation by producing IL-10 [11], AAMacs induction by type 2 cytokines might be one of the reasons underlying the improvement in the diabetic condition. Figure 3 shows significant elevation of IL-4, IL-13 and IL-10 and accumulation of a large number of AAMacs in the submucosa in the small intestine in the Hp-infected group. Metabolism is closely linked to the function of the immune cells. In particular, the activity of macrophages plays a key role in obesity and diabetes. Several previous researches described that it is important to maintain AAMacs in adipose tissues to maintain metabolic homeostasis. Liu *et al*. summarized the possible mechanisms by which IL-4 prevents T1D [21]. IL-4 may use several pathways, including the induction of T regulatory cells (Tregs) and AAMacs, and inhibit Th1 cytokines and CCR5 expression in pancreatic islets, in turn inhibiting T1D. Wu *et al*. showed strong evidence that eosinophils are the principal source of IL4 in adipose tissues to sustain AAMacs [51]. A similar mechanism might be applicable in the present study. We found several eosinophils accumulated in the submucosa in Hp-infected diabetic mice intestine (Fig. 4). These intestinal eosinophils might migrate to adipose tissues to sustain AAMacs. Moreover, Stanya *et al*. identified a metabolic role for IL-13 in the control of hepatic glucose production in a non-diabetic mice model. IL-13 inhibits transcription of gluconeogenic genes by acting directly on hepatocytes through Stat3 [43]. Interestingly, this function is independent of insulin signaling. Furthermore, there are new insights that intestinal helminthes modulate the immune system through alterations to the microbiota of the intestine. Zaiss *et al.* showed that helminthes infection attenuated the allergic inflammation by a specific reduction in infiltration

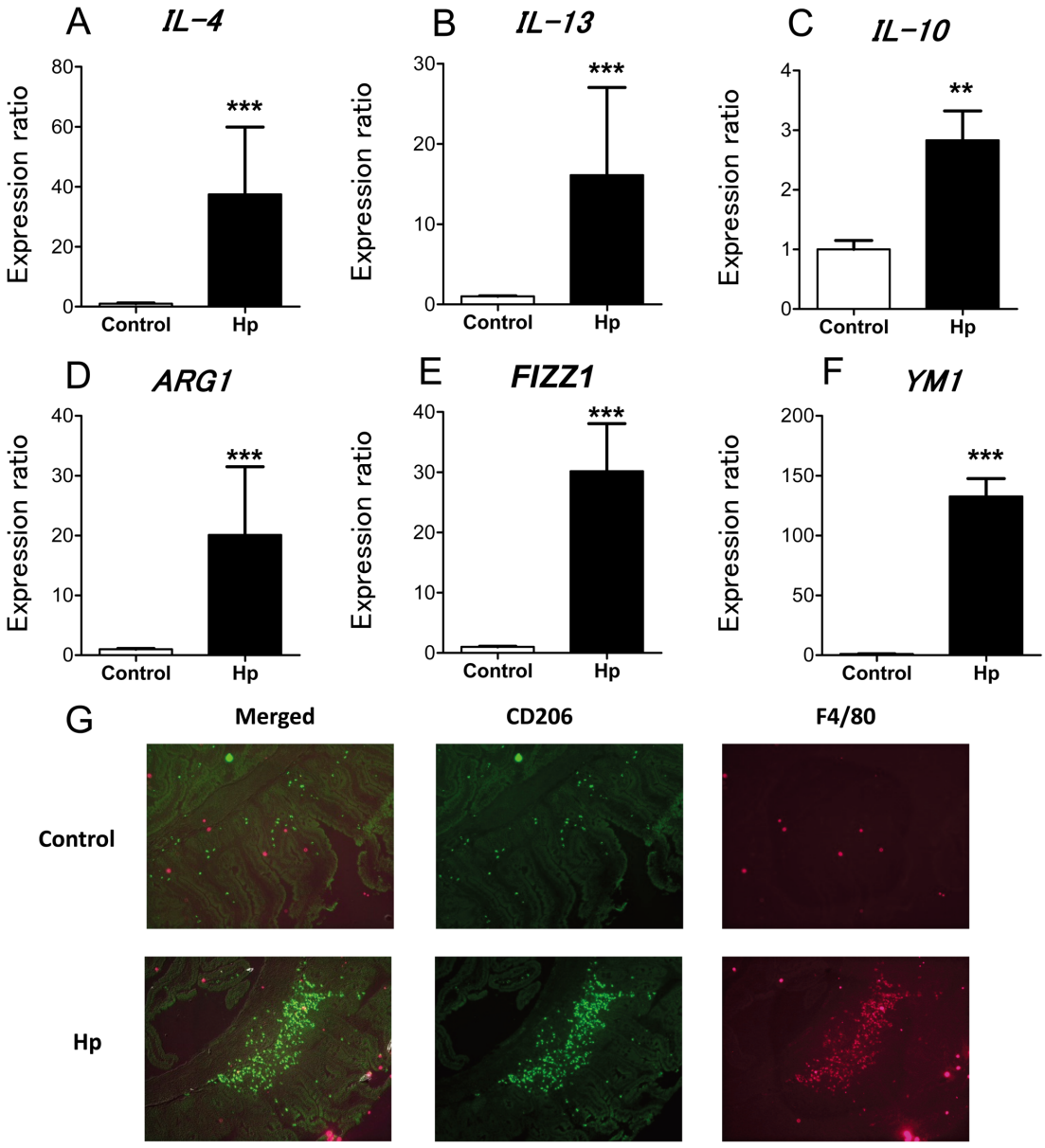

Fig. 3. Real-time PCR analysis of IL-4 (A), IL-13 (B), IL-10 (C), ARG1 (D), FIZZ1 (E) and YM1 (F) gene expression in the small intestine from uninfected control mice (white bars) and 8 days after Hp infection (black bars) of the study mice (12 weeks old, N=5). All data are expressed in relative units compared with uninfected mice (controls). $^{**}P<0.01$; $^{***}P<0.001$. Data are shown as means ± SE and represent two independent experiments with similar results. (G) Immunofluorescence staining with anti-CD206 (green) and anti-F4/80 (red) antibodies of a section of the small intestine. The observation that CD206+ cells were F4/80+ is consistent with AAMacs phenotype. Images left to right: merged FITC and Alexa647, FITC only, and Alexa647 only. All images are of 4-μm-thick sections at 100× magnification, and images represent submucosa obtained from more than five mice.

eosinophils of the lungs [53]. The ability of helminthes to modulate host's immune system should be examined more in the detail.

Another answer might be a decline in glucose absorption from the small intestine. Here, we focused on the glucose transporters in the epithelium. Our results suggested that the gene expression of GLUT2 was significantly decreased in the small intestine after infection. The details of the mechanism is still unclear, but Notari *et al*. presented a model for changes in epithelial glucose handling in response to *Nippostrongylus brasiliensis* (Nb) infection in BALB/c mice [34]. This mouse strain is not a diabetic model, but the authors pointed out that the downregulation of SGLT1 after Nb infection is dependent on AAMacs (M2) but not on GLUT2. However, in this study, Hp-infected KK-Ay/TaJcl diabetic mice did not show any differences in SGLT1 gene

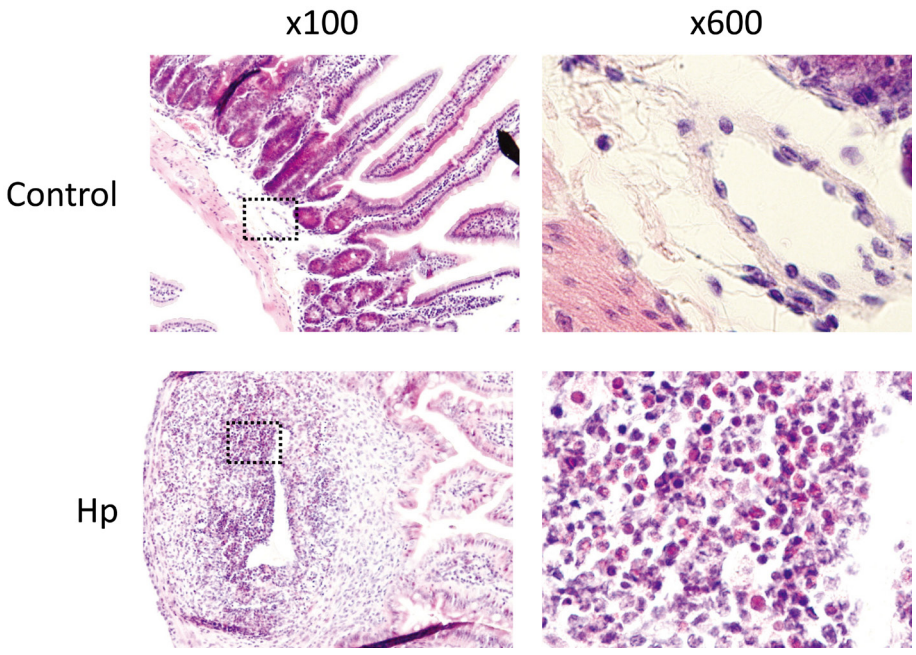

Fig. 4. Representative H&E stained sections in the small intestine (12 weeks old, control: top and day 8 after Hp infection: bottom). All images are 7-μm-thick sections at 100× (left) and 600× (right) magnification. Enlarged regions of the submucosa, indicated by a dashed rectangle, are shown in the left panel.

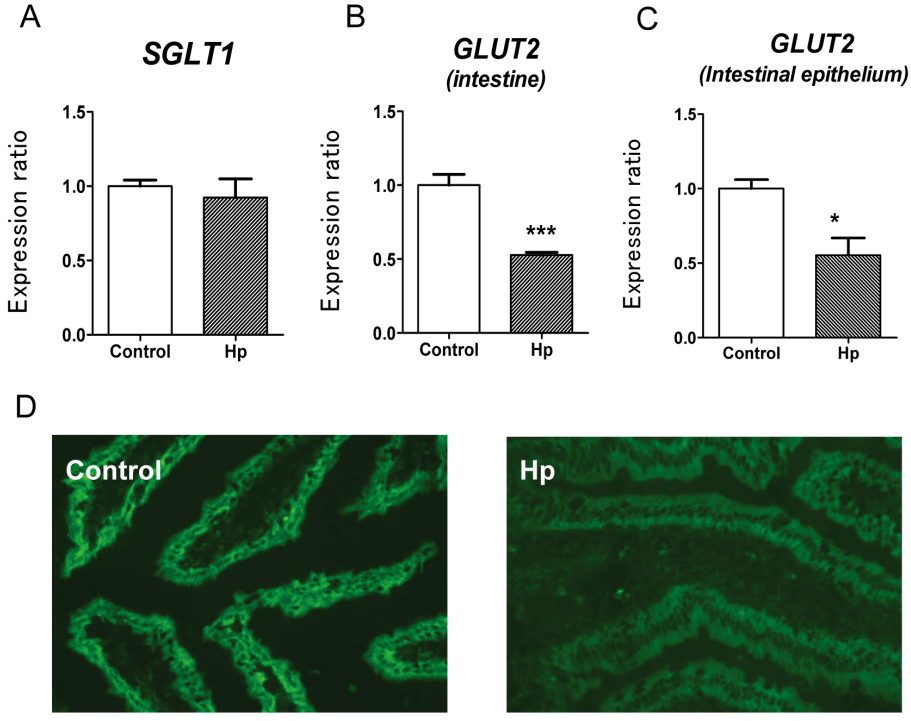

Fig. 5. (A) Real-time PCR analysis of SGLT1 in whole tissue of the small intestine. (B) Real-time PCR analysis of GLUT2 in whole tissue of the small intestine. (C) Real-time PCR analysis of GLUT2 in the epithelium of the small intestine with LCM. (D) Immunofluorescence staining of a section of the small intestine from control and Hp-infected mice (12 weeks old, day 8 after infection) with anti-GLUT2 antibody. *P<0.05; ***P<0.001. N≥4. Data are shown as means ± SE and represent two independent experiments with similar results.

expression after Hp infection (Fig. 5A). It is not clear why our results were not consistent with Notari's report, but these two nematodes differ slightly in terms of infection modus. Primary inoculation of mice with Hp is associated with chronic infection, while Nb infection is cleared acutely within 2 weeks after primary inoculation. In addition, Hp invades the host submucosa and forms a worm cyst, whereas Nb is retained in the gastrointestinal tract. Nevertheless, nematode infection changes the expression of GLUT2 in the small intestine, resulting in low glucose absorption. As mentioned in the beginning, Madden *et al*. showed that glucose absorption through the intestinal epithelium was decreased after nematode infection [23]. Alteration of glucose transporters in the small intestine by infection might be contributed to decline in glucose transportation in mice. The mechanism underlying the decline of GLUT2 after Hp infection is not defined. Notari *et al*. showed that the downregulation of GLUT2 was AAMacs independent, as mentioned above. However, previous reports described that Ca^{2+} [27] and taste receptors [22] mediate the regulation of GLUT2. Hp infection may change these factors by altering the epithelial conditions. Hepatic steatosis is another problem for T2D patients. Patients with T2D have a 44% lower rate of hepatic glycogen synthesis after meal ingestion than non-diabetic individuals, resulting in excessive postprandial hyperglycemia [39]. Elevated hepatocellular lipid levels mainly account for hepatic insulin resistance. Therefore, liver fat is believed to be an important therapeutic target in insulin resistance and T2D. We found that fatty liver condition improved markedly in Hp-infected diabetic mice. The mechanism is not clear, but inhibition of FAS gene expression by Hp infection might—at least in part—contribute to the improvement of liver condition. Previous studies have reported on the effect of agents against hepatic steatosis inhibiting FAS enzyme activity [18].

In conclusion, nematode infection appears to provide an effective option for the treatment of T2D by improving inflammatory status through restoration of the cytokine imbalance, inhibition of glucose absorption from the small intestine and decline of excess fat accumulation in the liver. To define the role of each of the immune cells during the interaction of the immunity and metabolic systems, it would be necessary to assess the effect of Th2, AAMac (M2) or eosinophil depletion; however, this was difficult in our model. We need future investigations to understand the details underlying the mechanism. We may gain a new therapeutic strategy against T2D by further analysis of nematode-induced immune- and non-immune–induced alteration of host function.

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REFERENCES

1. Anthony, R. M., Urban, J. F. Jr., Alem, F., Hamed, H. A., Rozo, C. T., Boucher, J. L., Van Rooijen, N. and Gause, W. C. 2006. Memory T(H)2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. *Nat. Med.* **12**: 955–960. [Medline] [CrossRef]
2. Chizzolini, C., Dayer, J. M. and Miossec, P. 2009. Cytokines in chronic rheumatic diseases: is everything lack of homeostatic balance? *Arthritis Res. Ther.* **11**: 246. [Medline] [CrossRef]
3. Clynes, R. 2010. B cell therapy is β-tested: Rituximab puts a pause on β-cell destruction. *Islets* **2**: 130–132. [Medline] [CrossRef]
4. Cooke, A., Tonks, P., Jones, F. M., O'Shea, H., Hutchings, P., Fulford, A. J. and Dunne, D. W. 1999. Infection with Schistosoma mansoni prevents insulin dependent diabetes mellitus in non-obese diabetic mice. *Parasite Immunol.* **21**: 169–176. [Medline] [CrossRef]
5. Folch, J., Lees, M. and Sloane Stanley, G. H. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226**: 497–509. [Medline]
6. Foryst-Ludwig, A., Hartge, M., Clemenz, M., Sprang, C., Hess, K., Marx, N., Unger, T. and Kintscher, U. 2010. PPARgamma activation attenuates T-lymphocyte-dependent inflammation of adipose tissue and development of insulin resistance in obese mice. *Cardiovasc. Diabetol.* **9**: 64. [Medline] [CrossRef]
7. Garg, A. 1998. High-monounsaturated-fat diets for patients with diabetes mellitus: a meta-analysis. *Am. J. Clin. Nutr.* **67** Suppl: 577S–582S. [Medline]
8. Garg, A., Bonanome, A., Grundy, S. M., Zhang, Z. J. and Unger, R. H. 1988. Comparison of a high-carbohydrate diet with a high-monounsaturated-fat diet in patients with non-insulin-dependent diabetes mellitus. *N. Engl. J. Med.* **319**: 829–834. [Medline] [CrossRef]
9. Garg, A., Bantle, J. P., Henry, R. R., Coulston, A. M., Griver, K. A., Raatz, S. K., Brinkley, L., Chen, Y. D., Grundy, S. M., Huet, B. A. and Reaven, G. M. 1994. Effects of varying carbohydrate content of diet in patients with non-insulin-dependent diabetes mellitus. *JAMA* **271**: 1421–1428. [Medline] [CrossRef]
10. Giulietti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R. and Mathieu, C. 2001. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* **25**: 386–401. [Medline] [CrossRef]
11. Gordon, S. 2003. Alternative activation of macrophages. *Nat. Rev. Immunol.* **3**: 23–35. [Medline] [CrossRef]
12. Gouyon, F., Caillaud, L., Carriere, V., Klein, C., Dalet, V., Citadelle, D., Kellett, G. L., Thorens, B., Leturque, A. and Brot-Laroche, E. 2003. Simple-sugar meals target GLUT2 at enterocyte apical membranes to improve sugar absorption: a study in GLUT2-null mice. *J. Physiol.* **552**: 823–832. [Medline] [CrossRef]
13. Heilbronn, L. K. and Campbell, L. V. 2008. Adipose tissue macrophages, low grade inflammation and insulin resistance in human obesity. *Curr. Pharm. Des.* **14**: 1225–1230. [Medline] [CrossRef]
14. Hong, E. G., Ko, H. J., Cho, Y. R., Kim, H. J., Ma, Z., Yu, T. Y., Friedline, R. H., Kurt-Jones, E., Finberg, R., Fischer, M. A., Granger, E. L., Norbury, C. C., Hauschka, S. D., Philbrick, W. M., Lee, C. G., Elias, J. A. and Kim, J. K. 2009. Interleukin-10 prevents diet-induced insulin resistance by attenuating macrophage and cytokine response in skeletal muscle. *Diabetes* **58**: 2525–2535. [Medline] [CrossRef]
15. Hung, T., Sievenpiper, J. L., Marchie, A., Kendall, C. W. and Jenkins, D. J. 2003. Fat versus carbohydrate in insulin resistance, obesity, diabetes and cardiovascular disease. *Curr. Opin. Clin. Nutr. Metab. Care* **6**: 165–176. [Medline] [CrossRef]
16. Kang, K., Reilly, S. M., Karabacak, V., Gangl, M. R., Fitzgerald, K., Hatano, B. and Lee, C. H. 2008. Adipocyte-derived Th2 cytokines and myeloid PPARdelta regulate macrophage polariza-

tion and insulin sensitivity. *Cell Metab.* **7**: 485–495. [Medline] [CrossRef]
17. Kitagaki, K., Businga, T. R., Racila, D., Elliott, D. E., Weinstock, J. V. and Kline, J. N. 2006. Intestinal helminths protect in a murine model of asthma. *J. Immunol.* **177**: 1628–1635. [Medline] [CrossRef]
18. Lelliott, C. J., López, M., Curtis, R. K., Parker, N., Laudes, M., Yeo, G., Jimenez-Liñan, M., Grosse, J., Saha, A. K., Wiggins, D., Hauton, D., Brand, M. D., O'Rahilly, S., Griffin, J. L., Gibbons, G. F. and Vidal-Puig, A. 2005. Transcript and metabolite analysis of the effects of tamoxifen in rat liver reveals inhibition of fatty acid synthesis in the presence of hepatic steatosis. *FASEB J.* **19**: 1108–1119. [Medline] [CrossRef]
19. Leung, J., Hang, L., Blum, A., Setiawan, T., Stoyanoff, K. and Weinstock, J. 2012. Heligmosomoides polygyrus abrogates antigen-specific gut injury in a murine model of inflammatory bowel disease. *Inflamm. Bowel Dis.* **18**: 1447–1455. [Medline] [CrossRef]
20. Liu, Q., Sundar, K., Mishra, P. K., Mousavi, G., Liu, Z., Gaydo, A., Alem, F., Lagunoff, D., Bleich, D. and Gause, W. C. 2009. Helminth infection can reduce insulitis and type 1 diabetes through CD25- and IL-10-independent mechanisms. *Infect. Immun.* **77**: 5347–5358. [Medline] [CrossRef]
21. Liu, Z., Liu, Q., Bleich, D., Salgame, P. and Gause, W. C. 2010. Regulation of type 1 diabetes, tuberculosis, and asthma by parasites. *J. Mol. Med.* **88**: 27–38. [Medline] [CrossRef]
22. Mace, O. J., Affleck, J., Patel, N. and Kellett, G. L. 2007. Sweet taste receptors in rat small intestine stimulate glucose absorption through apical GLUT2. *J. Physiol.* **582**: 379–392. [Medline] [CrossRef]
23. Madden, K. B., Yeung, K. A., Zhao, A., Gause, W. C., Finkelman, F. D., Katona, I. M., Urban, J. F. Jr. and Shea-Donohue, T. 2004. Enteric nematodes induce stereotypic STAT6-dependent alterations in intestinal epithelial cell function. *J. Immunol.* **172**: 5616–5621. [Medline] [CrossRef]
24. Maizels, R. M., Hewitson, J. P., Murray, J., Harcus, Y. M., Dayer, B., Filbey, K. J., Grainger, J. R., McSorley, H. J., Reynolds, L. A. and Smith, K. A. 2012. Immune modulation and modulators in Heligmosomoides polygyrus infection. *Exp. Parasitol.* **132**: 76–89. [Medline] [CrossRef]
25. Matthews, D. R., Hosker, J. P., Rudenski, A. S., Naylor, B. A., Treacher, D. F. and Turner, R. C. 1985. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* **28**: 412–419. [Medline] [CrossRef]
26. Moon, J. H., Lee, J. Y., Kang, S. B., Park, J. S., Lee, B. W., Kang, E. S., Ahn, C. W., Lee, H. C. and Cha, B. S. 2010. Dietary monounsaturated fatty acids but not saturated fatty acids preserve the insulin signaling pathway via IRS-1/PI3K in rat skeletal muscle. *Lipids* **45**: 1109–1116. [Medline] [CrossRef]
27. Morgan, E. L., Mace, O. J., Affleck, J. and Kellett, G. L. 2007. Apical GLUT2 and Cav1.3: regulation of rat intestinal glucose and calcium absorption. *J. Physiol.* **580**: 593–604. [Medline] [CrossRef]
28. Morimoto, M., Morimoto, M., Whitmire, J. and Star, R. A., Urban, J. F., Jr. and Gause, W. C. 2003. In situ localization of gene expression using laser capture microdissection. pp. 135–147. *In*: PCR Primer, 2nd ed. (C.W., Dieffenbach and G.S., Dveksler. eds.), Cold Spring Harbor Laboratory Press, New York.
29. Morimoto, M., Morimoto, M., Whitmire, J., Xiao, S., Anthony, R. M., Mirakami, H., Star, R. A., Urban, J. F. Jr. and Gause, W. C. 2004. Peripheral CD4 T cells rapidly accumulate at the host: parasite interface during an inflammatory Th2 memory response. *J. Immunol.* **172**: 2424–2430. [Medline] [CrossRef]
30. Morimoto, M., Morimoto, M., Zhao, A., Madden, K. B., Dawson, H., Finkelman, F. D., Mentink-Kane, M., Urban, J. F. Jr., Wynn, T. A. and Shea-Donohue, T. 2006. Functional importance of regional differences in localized gene expression of receptors for IL-13 in murine gut. *J. Immunol.* **176**: 491–495. [Medline] [CrossRef]
31. Morimoto, M., Saito, C., Muto, C., Akamatsu, Y., Chiba, T., Abe, T., Azuma, N. and Suto, Y. 2015. Impairment of host resistance to helminthes with age in murine small intestine. *Parasite Immunol.* **37**: 171–179. [Medline] [CrossRef]
32. Müller, B. 2002. Cytokine imbalance in non-immunological chronic disease. *Cytokine* **18**: 334–339. [Medline] [CrossRef]
33. Nishimura, M. 1969. Breeding of Mice Strains for Diabetes Mellitus. *Exp. Anim.* **18**: 147–157.
34. Notari, L., Riera, D. C., Sun, R., Bohl, J. A., McLean, L. P., Madden, K. B., van Rooijen, N., Vanuytsel, T., Urban, J. F. Jr., Zhao, A. and Shea-Donohue, T. 2014. Role of macrophages in the altered epithelial function during a type 2 immune response induced by enteric nematode infection. *PLOS ONE* **9**: e84763. [Medline] [CrossRef]
35. Odegaard, J. I., Ricardo-Gonzalez, R. R., Goforth, M. H., Morel, C. R., Subramanian, V., Mukundan, L., Red Eagle, A., Vats, D., Brombacher, F., Ferrante, A. W. and Chawla, A. 2007. Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. *Nature* **447**: 1116–1120. [Medline] [CrossRef]
36. Odegaard, J. I., Ricardo-Gonzalez, R. R., Red Eagle, A., Vats, D., Morel, C. R., Goforth, M. H., Subramanian, V., Mukundan, L., Ferrante, A. W. and Chawla, A. 2008. Alternative M2 activation of Kupffer cells by PPARdelta ameliorates obesity-induced insulin resistance. *Cell Metab.* **7**: 496–507. [Medline] [CrossRef]
37. Parillo, M., Rivellese, A. A., Ciardullo, A. V., Capaldo, B., Giacco, A., Genovese, S. and Riccardi, G. 1992. A high-monounsaturated-fat/low-carbohydrate diet improves peripheral insulin sensitivity in non-insulin-dependent diabetic patients. *Metabolism* **41**: 1373–1378. [Medline] [CrossRef]
38. Plomgaard, P., Bouzakri, K., Krogh-Madsen, R., Mittendorfer, B., Zierath, J. R. and Pedersen, B. K. 2005. Tumor necrosis factor-alpha induces skeletal muscle insulin resistance in healthy human subjects via inhibition of Akt substrate 160 phosphorylation. *Diabetes* **54**: 2939–2945. [Medline] [CrossRef]
39. Roden, M. 2006. Mechanisms of Disease: hepatic steatosis in type 2 diabetes--pathogenesis and clinical relevance. *Nat. Clin. Pract. Endocrinol. Metab.* **2**: 335–348. [Medline] [CrossRef]
40. Sell, H., Habich, C. and Eckel, J. 2012. Adaptive immunity in obesity and insulin resistance. *Nat. Rev. Endocrinol.* **8**: 709–716. [Medline] [CrossRef]
41. Sewell, D., Qing, Z., Reinke, E., Elliot, D., Weinstock, J., Sandor, M. and Fabry, Z. 2003. Immunomodulation of experimental autoimmune encephalomyelitis by helminth ova immunization. *Int. Immunol.* **15**: 59–69. [Medline] [CrossRef]
42. Smith, P., Mangan, N. E., Walsh, C. M., Fallon, R. E., McKenzie, A. N., van Rooijen, N. and Fallon, P. G. 2007. Infection with a helminth parasite prevents experimental colitis via a macrophage-mediated mechanism. *J. Immunol.* **178**: 4557–4566. [Medline] [CrossRef]
43. Stanya, K. J., Jacobi, D., Liu, S., Bhargava, P., Dai, L., Gangl, M. R., Inouye, K., Barlow, J. L., Ji, Y., Mizgerd, J. P., Qi, L., Shi, H., McKenzie, A. N. and Lee, C. H. 2013. Direct control of hepatic glucose production by interleukin-13 in mice. *J. Clin. Invest.* **123**: 261–271. [Medline] [CrossRef]

44. Summers, R. W., Elliott, D. E., Urban, J. F. Jr., Thompson, R. A. and Weinstock, J. V. 2005. Trichuris suis therapy for active ulcerative colitis: a randomized controlled trial. *Gastroenterology* **128**: 825–832. [Medline] [CrossRef]
45. Summers, R. W., Elliott, D. E., Urban, J. F. Jr., Thompson, R. and Weinstock, J. V. 2005. Trichuris suis therapy in Crohn's disease. *Gut* **54**: 87–90. [Medline] [CrossRef]
46. Talal, N. and Flescher, E. 1988. Rheumatoid arthritis: an editorial perspective based on cytokine imbalance. *J. Autoimmun.* **1**: 309–317. [Medline] [CrossRef]
47. Taylor, M. D., van der Werf, N. and Maizels, R. M. 2012. T cells in helminth infection: the regulators and the regulated. *Trends Immunol.* **33**: 181–189. [Medline] [CrossRef]
48. Urban, J. F. Jr., Katona, I. M., Paul, W. E. and Finkelman, F. D. 1991. Interleukin 4 is important in protective immunity to a gastrointestinal nematode infection in mice. *Proc. Natl. Acad. Sci. U.S.A.* **88**: 5513–5517. [Medline] [CrossRef]
49. Winer, S., Chan, Y., Paltser, G., Truong, D., Tsui, H., Bahrami, J., Dorfman, R., Wang, Y., Zielenski, J., Mastronardi, F., Maezawa, Y., Drucker, D. J., Engleman, E., Winer, D. and Dosch, H. M. 2009. Normalization of obesity-associated insulin resistance through immunotherapy. *Nat. Med.* **15**: 921–929. [Medline] [CrossRef]
50. Wright, E. M., Hirayama, B. A. and Loo, D. F. 2007. Active sugar transport in health and disease. *J. Intern. Med.* **261**: 32–43. [Medline] [CrossRef]
51. Wu, D., Molofsky, A. B., Liang, H. E., Ricardo-Gonzalez, R. R., Jouihan, H. A., Bando, J. K., Chawla, A. and Locksley, R. M. 2011. Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis. *Science* **332**: 243–247. [Medline] [CrossRef]
52. Yang, Z. H., Miyahara, H. and Hatanaka, A. 2011. Chronic administration of palmitoleic acid reduces insulin resistance and hepatic lipid accumulation in KK-Ay Mice with genetic type 2 diabetes. *Lipids Health Dis.* **10**: 120. [Medline] [CrossRef]
53. Zaiss, M. M., Rapin, A., Lebon, L., Dubey, L. K., Mosconi, I., Sarter, K., Piersigilli, A., Menin, L., Walker, A. W., Rougemont, J., Paerewijck, O., Geldhof, P., McCoy, K. D., Macpherson, A. J., Croese, J., Giacomin, P. R., Loukas, A., Junt, T., Marsland, B. J. and Harris, N. L. 2015. The Intestinal Microbiota Contributes to the Ability of Helminths to Modulate Allergic Inflammation. *Immunity* **43**: 998–1010. [Medline] [CrossRef]
54. Zhao, A., Urban, J. F., Jr., Anthony, R. M., Sun, R., Stiltz, J., van Rooijen, N., Wynn, T. A., Gause, W. C. and Shea-Donohue, T. 2008. Th2 cytokine-induced alterations in intestinal smooth muscle function depend on alternatively activated macrophages. *Gastroenterology* **135**: 217–225 e211. [Medline] [CrossRef]