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High fat diet induces alterations to intraepithelial lymphocyte and cytokine mRNA in the small intestine of C57BL/6 mice

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The aim of this work was to investigate the possible effect of high fat diet (HFD) induced obesity on iIEL subsets and their cytokine mRNA levels in C57BL/6 mice. Two groups of C57BL/6 mice were kept in separate cages and fed with either a normal diet (control) or HFD. After 12 weeks of treatment the iIELs were isolated and analyzed by FACS. Additionally, the mRNA levels of IL-2, IL-15, TGF- β , TNF- α , IFN- γ granzyme B and perforin were quantified by RT-PCR. We found that HFD induced a proportional shift in TCR $\gamma\delta^+$ and TCR $\alpha\beta^+$ composition within the iIEL population. Also, a significant increase in the mRNA levels of TNF- α , perforin and granzyme B was detected on iIELs from HFD mice. Our findings may provide important insights into intestinal changes associated to HFD induced obesity, which could eventually lead to intestinal inflammation. However, further investigations are required to elucidate the molecular mechanism of HFD induced changes on iIELs.

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Introduction

Worldwide, obesity is one of the most important public health problems. It has been described as a chronic degenerative disease, produced by an imbalance between intake and energy consumption, with contribution of environmental and genetic factors.^{1,2} Obesity is associated with type 2 diabetes mellitus, enhanced cardiovascular risk, fatty liver, and others metabolic alterations.^{3–5} Moreover, obesity is strongly linked to chronic low grade systemic inflammation, characterized by increased levels of IL-6, TNF- α and acute phase proteins.^{6–8} Although the mechanisms underlying the inflammation associated with obesity have not been entirely elucidated, it is well known that adipose tissue is an important source of pro-inflammatory cytokines.⁹ In addition, biological pro inflammatory mechanisms in gut could also contribute to this phenomenon.^{10,11}

On the other hand, intestinal intraepithelial lymphocytes (iIELs) are an essential mechanism of cellular defense against pathogenic microorganisms. The iIELs have also important immunoregulatory functions, and a potent cytolytic activity.^{12–15} In mice, similar proportions of TCR $\alpha\beta$ and TCR $\gamma\delta$ expressing

iIELs have been identified in the intestinal compartment, most of these cells are expressing the homodimer CD8 $\alpha\alpha$, which has not been detected on other tissues.^{16–19} Likewise, CD4[–]CD8[–] double negative lymphocytes reside in the small intestine.²⁰ Furthermore, CD4⁺ lymphocytes are a minor subset in the iIELs compartment, and some of these cells are CD4⁺CD8⁺ double positive.^{21,22} In addition, iIELs include thymus-dependent cells such as TCR $\alpha\beta^+$ CD8 $\alpha\beta^+$, as well as thymus-independent cells; such as both TCR $\alpha\beta^+$ CD8 $\alpha\alpha^+$ and TCR $\gamma\delta^+$ CD8 $\alpha\alpha^+$.^{23–25}

iIELs may regulate intestinal functions by producing cytokines, which control intestinal homeostasis and injury. Under pathological environment, iIELs cytokine expression is altered, and these changes could mediate functional modifications in the small intestine. iIELs secrete mainly IFN- γ , TNF- α , IL-2, TGF- β , IL15 and others. This way, cytokines produced by iIELs are an important way to modulate the local intestinal immune response, as well as keep the integrity of the intestinal barrier.²⁶

iIELs development is dependent of different factors, including intestinal microbiota,^{27,28} specific TCR engagement,^{29,30} and vitamin D.³¹ On the other hand, it has been reported that high fat diet (HFD) is associated with important changes in gut microbiota, triggering an inflammatory response;^{11,32} however, the possible changes on the iIELs subsets have not been explored on HFD induced obesity mice model. Herein, the effect of HFD on iIELs subsets, as well as mRNA cytokines levels in C57BL/6 mice was determined. We found that HFD induced significant changes in iIEL subsets, together with an increase on mRNA levels of TNF- α , perforin, and granzyme-B. Our data suggest that HFD lead to disrupt the intestinal homeostasis, producing changes on iIELs

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proportions and increasing mRNA levels of TNF- α , perforin, granzyme-B. Thus, HFD induced intestinal changes may contribute to trigger the inflammation linked to obesity.

Material and methods

Mice and antibodies

Five to seven week old C57BL/6NHsd male mice were purchased from Harlan Mexico, S.A. de C.V. (México, D.F.). Mice were maintained in a pathogen-free environment, under standard room conditions of temperature, humidity and free access to food and water. The mice were maintained in accordance with to the official Mexican guidelines for the use and care of experimental animals, and the protocol was approved by the ethics committee of the Department of Medical Sciences of Universidad de Guanajuato, México.

The following monoclonal antibodies were used for iIELs staining and FACS analysis: FITC-anti-CD8 β , PE-anti-TCR $\gamma\delta$, PerCP-anti-CD8 α , APC-anti-TCR β , PerCP-anti-CD4, and purified anti-Fc γ receptor (CD16/CD32.). All anti mouse monoclonal antibodies were purchased from BD Biosciences (San Diego, CA), and diluted 1 : 10 in FACS buffer (PBS, FBS 5%, and sodium azide 0.05%) before use them.

Diet intervention

Two groups of mice ($n = 10$ each group) were kept in separate cages. One of these designated as a control, fed with a normal diet (2014S Teklad Global 14% protein rodent maintenance diet, 4% fat); whereas another group was fed with a high fat diet for 12 weeks (TD.06414, adjusted calories diet: 60 kcal/fat, 34.3% fat, 27.3% protein). According to the diet manufacturer, the approximate fatty acid profile (% of total fat) is the following: 37% saturated, 47% monounsaturated, 16% polyunsaturated. Both diets were purchased from Harlan Mexico S.A. de C.V. After 12 weeks, mice were sacrificed and the small intestine was removed to isolate and purified the iIELs.

Body weight gain and glucose, cholesterol, and triglyceride serum levels measurement

The weight gain of each mouse was assessed with an analytical balance and recorded every 7 days until the end of the experiment. Blood samples were obtained from the retro-ocular plexus with a capillary microtube and immediately used for glucose, cholesterol and triglyceride determinations, with an Accutrend GCT system (according to the manufacturer's instructions). After that, mice were sacrificed by cervical dislocation and small intestine was collected in cold PBS.

iIELs purification and staining

iIELs were isolated from the small intestine as previously described.³³ Briefly, Peyer's patches were identified and removed. After flushing with 50 mL of PBS (4 °C), gut was opened on a wet linen square. The mucosa was scraped with a scalpel and then dissociated by stirring for 20 min at room temperature in 50 mL of RPMI 1640 containing 10% heat inactivated FBS plus 1 mM dithioerythritol (Sigma Chemical Co., St. Louis, MO) and 1 mM

EDTA. After centrifugation at 1500 rpm for 10 min, the cell pellet was resuspended in RPMI 1640 and passed through a glass wool column prewashed with RPMI 1640 containing 5 mM HEPES. The suspension obtained was then centrifuged and the pellet used to purify iIELs by centrifugation through a discontinuous Percoll (Amersham Pharmacia Biotech, Piscataway, NJ) gradient. Cells were resuspended in a 72% Percoll/PBS solution, overlaid with an equal volume of 36% Percoll/PBS and centrifuged at 2000 $\times g$ for 30 min at room temperature. Then, cells at the interface (iIELs) were, washed and incubated with anti-CD16/CD32 to block nonspecific mAb binding. Cells were then immunostained with different combinations of the indicated mAbs. Four-color multiparameter immunofluorescence staining was analyzed in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). iIELs were gated using forward and side scatter to exclude dead cells, and the data were analyzed using the Cell Quest software (Becton Dickinson, San Jose, CA).

Measurement of cytokine mRNA levels by RT-PCR

Total mRNA was isolated from iIELs using the RNeasy Mini Kit (QIAGEN, Valencia CA), each RNA sample (2 ng) was reverse-transcribed with 50 μM of oligo(dT)₂₀, 10 mM dNTP mix, 0.1 M DTT, and 15 U μL^{-1} of ThermoScript™ RT-PCR system (Invitrogen, Alameda CA) in a volume of 20 μL at 42 °C for 60 min for synthesis of first-strand cDNA. The PCR amplification was processed in a Perkin Elmer Thermo Cycler 9700 as described above. The PCR products were subjected to electrophoresis in 1.8% agarose gels, stained with ethidium bromide and visualized under UV light. The specific oligonucleotide primers used in PCR amplification were as follows: IL-2 sense, 5'-TGA TGG ACC TAC AGG AGC TCC TGA G-3'; IL-2 antisense, 5'-GAG TCA AAT CCA GAA CAT GCC GCA G-3'; IL-15 sense, 5'-GGA AGG CTG AGT TCC ACA TC-3'; IL-15 antisense, 5'-AGG GAG ACC TAC ACT GAC AC-3'; IFN- γ sense, 5'-AGC GGC TGA CTG AAC TCA GAT TGT AGC TTG TAC CTT TAC TTC ACT G-3'; IFN- γ antisense, 5'-GTC ACA GTT TTC AGC TGT ATAGGG-3'; TNF- α sense, 5'-GGC AGG TCT ACT TTG GAG TCA TTG C-3'; TNF- α antisense, 5'-ACA TTC GAG GCT CCA GTG AAT TCG G-3'; TGF- β sense, 5'-TGG ACC GCA ACA ACG CCA TCT ATG-3'; TGF- β sense, 5'-TGG AGC TGA AGC AAT AGT TGG TAT CC-3'; granzyme B sense, 5'-GCC CAC AAC ATC AAA GAA CAG-3'; granzyme B antisense, 5'-AAC CAG CCA CAT AGC ACA CAT-3'; perforin sense, 5'-GTC ACG TCG AAG TAC TTG GTG-3'; perforin antisense, 5'-ATG GCT GAT AGC CTG TCT CAG-3'; β -actin sense, 5'-TGG, AAT CCT GTG GCA TCC ATG AAA C-3'; β -actin antisense, 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'. The β -actin was used as internal control.

Additionally, quantitative real-time polymerase chain reactions (qRT-PCR) were performed using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) on a StepOnePlus real-time PCR system (Applied Biosystems) following standard procedures. Briefly, cDNA was synthesized from 1 μg of total RNA using High cDNA Reverse Transcription Kit (Applied Biosystems). For PCR reactions, 1 μL of cDNA template, 0.3 μL of forward and reverse primers for specific cytokine (10 $\mu\text{mol L}^{-1}$ each), 10 μL of SYBR Green PCR Master Mix, and 8.4 μL of water were added to a final volume of 20 μL . Thermal cycling was



performed as follows: 95 °C for 3 minutes as initial denaturing, followed by 40 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds, and a final extension at 72 °C for 2 minutes. Threshold cycles (C_T values) were determined using the StepOne software (Applied Biosystems). Real-time PCR results were normalized using β -actin as an internal control. Relative mRNA levels were calculated from the C_T values for each sample.

Results

Effect of HFD on body weight and plasma triglycerides

After 12 weeks of treatment the body weight of HFD mice increased from 20.6 ± 2.4 to 42 ± 3.7 g, while a slight increase of body weight was observed in mice feed with normal diet (Fig. 1a). In addition, although serum levels of glucose and cholesterol were similar in both groups (Fig. 1b and c), a significant increase on triglycerides was detected in HFD mice (Fig. 1d).

Effect of HFD on TCR $\gamma\delta$ iIELs

We found that HFD induced a significant increase on the proportion of TCR $\gamma\delta$ iIELs (from 47 ± 3 to $67 \pm 7\%$, $p < 0.05$, Fig. 2a and b). Accordingly, a significant decrease on the proportion of TCR $\alpha\beta$ iIELs was detected in HFD mice (from 44 ± 4 to $23 \pm 5\%$, $p < 0.05$, Fig. 2). In contrast, no significant

changes on percentage of both TCR $\gamma\delta$ and TCR $\alpha\beta$ iIELs were observed in control mice (Fig. 2a and b).

HFD had not effect on iIELs expressing CD4, CD8 $\alpha\beta$ or CD8 $\alpha\alpha$

Additional flow cytometry analysis showed that TCR $\alpha\beta^+$ CD4 $^+$ cells were not affected by HFD, from $4.4 \pm 0.8\%$ in control to $4.3 \pm 1.0\%$ in HFD mice (Fig. 3a). Likewise, the proportions of both CD8 $\alpha\beta^+$ and CD8 $\alpha\alpha$ subsets gated on TCR $\alpha\beta^+$ iIELs were similar in HFD and control mice at the beginning and after 12 weeks of HFD. The percentage of iIELs expressing TCR $\alpha\beta^+$ CD8 $\alpha\beta^+$ was $31 \pm 1.9\%$ in control and $34 \pm 2\%$ in HFD mice; while the percentage of iIELs expressing TCR $\alpha\beta^+$ CD8 $\alpha\alpha^+$ was $55 \pm 4\%$ in control and $48 \pm 4\%$, in HFD mice, respectively (Fig. 3b). Similarly, no significant changes were observed in TCR $\gamma\delta$ lymphocytes expressing CD8 $\alpha\alpha$ or CD8 $\alpha\beta$ in either HFD or control mice. The percentage of iIELs expressing TCR $\gamma\delta^+$ CD8 $\alpha\alpha^+$ was $88 \pm 5\%$ in control and $85 \pm 3\%$ in HFD mice; while the percentage of iIELs expressing TCR $\gamma\delta^+$ CD8 $\alpha\beta$ was $3.4 \pm 4\%$, in control and $5.5 \pm 2\%$ in HFD mice, respectively (Fig. 4).

High fat diet induces changes in the pattern of cytokine synthesis by iIELs

To determine the possible effect of HFD on mRNA levels of different cytokines (IL-2, IL-15, TGF- β , TNF- α , IFN- γ) and cytotoxic molecules produced by iIELs, the mRNAs levels were

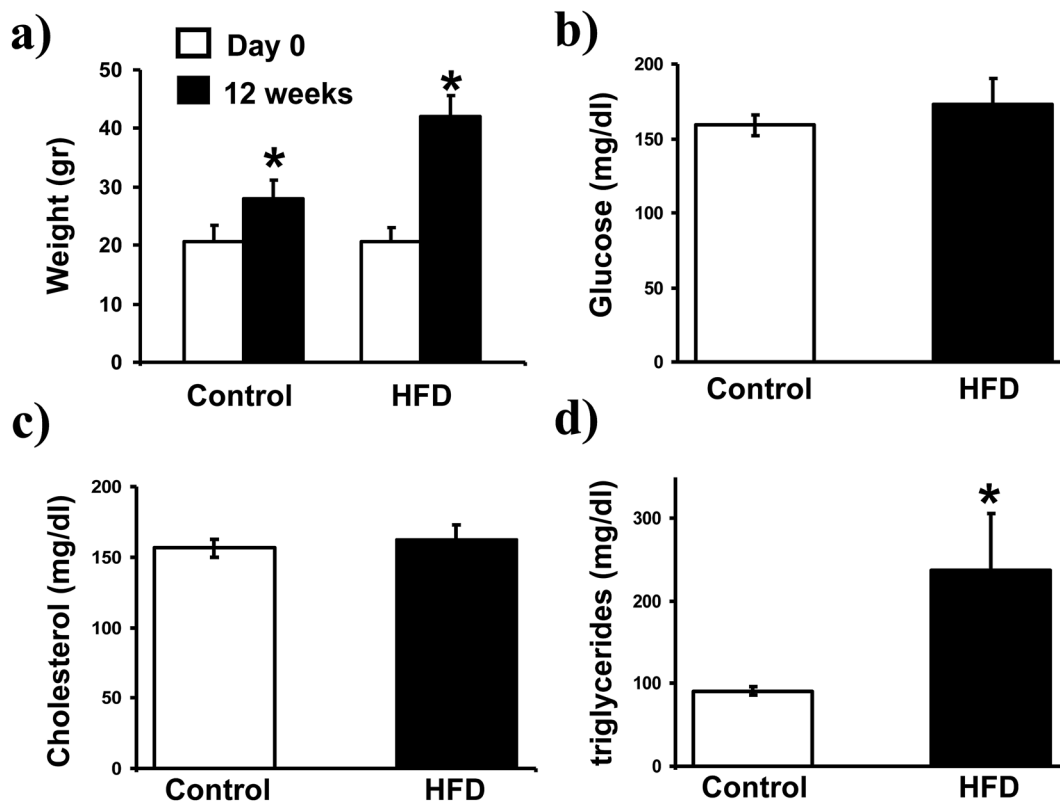


Fig. 1 Body weight gain and glucose, cholesterol, triglycerides levels on plasma. The body weight gain of each mouse was assessed and recorded at day 0 and 12 weeks of the experiment; graph bars in (a) represent the arithmetic mean \pm SD of weight in grams (g) of 10 mice. Blood samples were obtained from the retro-ocular plexus to determine glucose, cholesterol, and triglycerides levels with an Accutrend GCT system. The bars in the histograms represent the arithmetic mean \pm SD of glucose (b), cholesterol (c) and triglyceride (d) in mg dL $^{-1}$ of 10 mice. * $p < 0.05$ by U Mann Whitney.



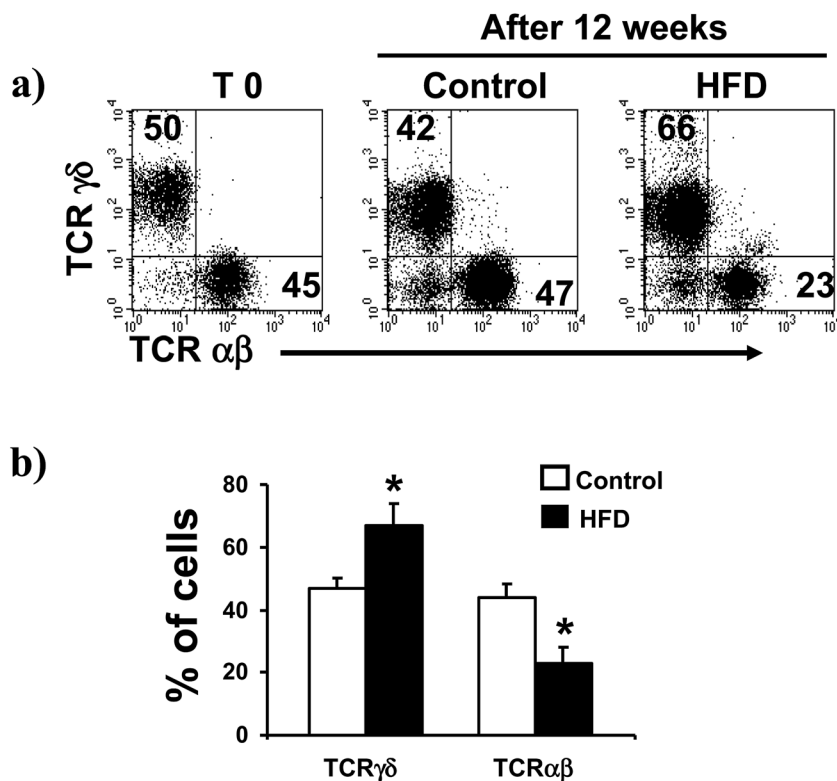


Fig. 2 TCR $\gamma\delta$ and TCR $\alpha\beta$ expressing cells in the small intestine of control and HFD feed mice. Freshly isolated iIELs were stained with anti TCR $\gamma\delta$, and TCR $\alpha\beta$ monoclonal antibodies and analyzed by flow cytometry. (a) Left panel correspond to representative flow cytometry dot plot of TCR $\gamma\delta$ and TCR $\alpha\beta$ expression on day 0, and middle and right panels correspond to 12 weeks of control and HFD mouse, respectively. (b) HFD induced a significant increase in the proportion of TCR $\gamma\delta$ iIELs, and a significant decrease on TCR $\alpha\beta$ iIELs. Results are the mean \pm SD of 10 mice in each group, * $p < 0.05$ compared with control mice by U Mann Whitney.

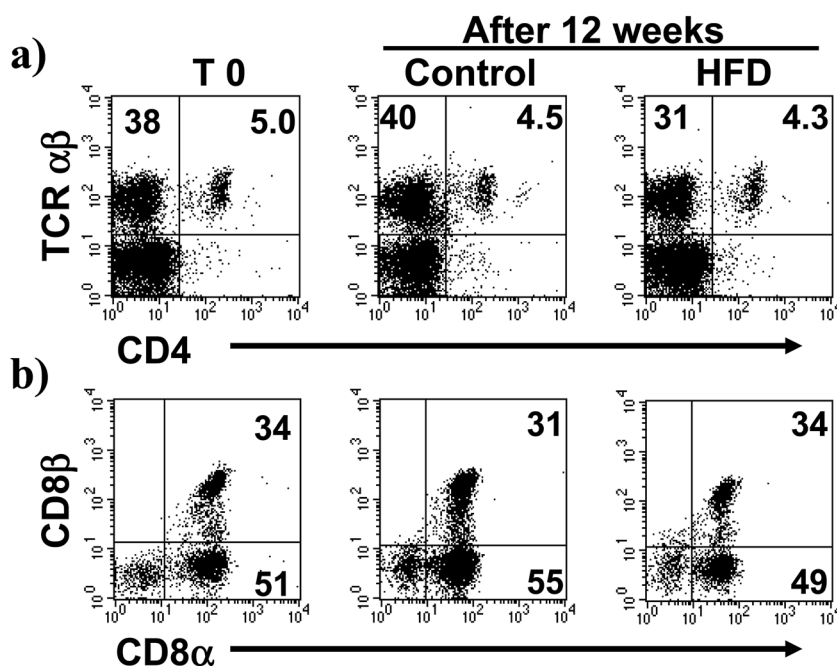


Fig. 3 Subsets on TCR $\alpha\beta$ ⁺ expressing cells in the small intestine of control and HFD feed mice. Freshly isolated iIELs were stained with anti TCR $\gamma\delta$, TCR $\alpha\beta$, CD8 α and CD8 β monoclonal antibodies and analyzed by flow cytometry. (a) Correspond to representative flow cytometry dot plot of TCR $\alpha\beta$ ⁺CD4⁺ expression on day 0 (left panel), and middle and right panels correspond to 12 weeks of control and HFD mice, respectively. (b) Correspond to representative flow cytometry dot plot of CD8 α and CD8 β expression iIELs gated on TCR $\alpha\beta$ ⁺ cells on day 0 (left panel), and middle and right panels correspond to 12 weeks of control and HFD mice, respectively.



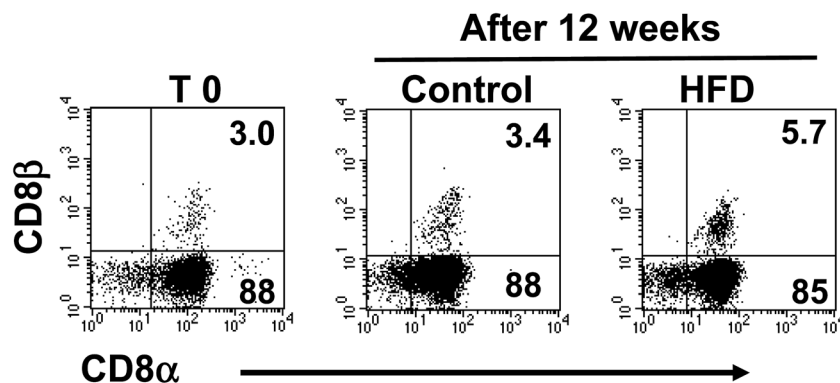


Fig. 4 Subsets on TCR $\delta\gamma^+$ expressing cells in the small intestine of control and HFD feed mice. Freshly isolated iIELs were stained with anti TCR $\gamma\delta$, TCR $\alpha\beta$, CD8 α and CD8 β monoclonal antibodies and analyzed by flow cytometry. Left panel corresponds to representative flow cytometry dot plot of CD8 α and CD8 β expression iIELs gated on TCR $\gamma\delta^+$ cells on day 0, and middle and right panels correspond to 12 weeks of control and HFD mice, respectively.

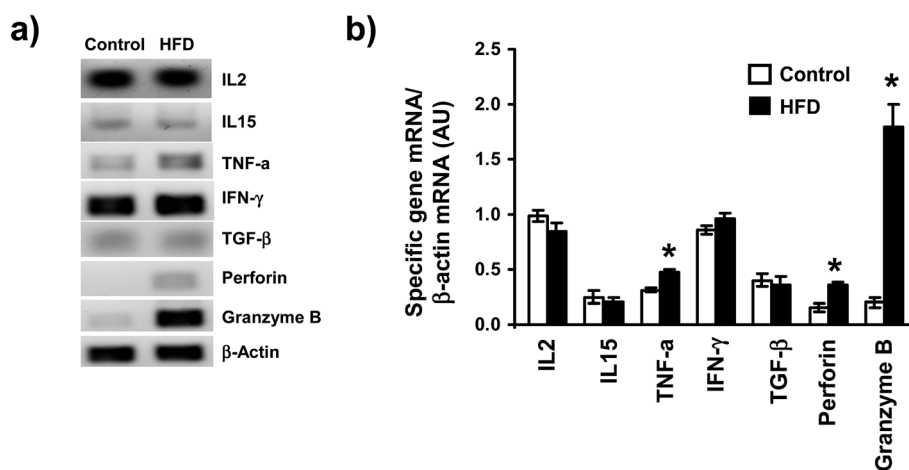


Fig. 5 Cytokines mRNA expression on total iIELs from ND and HFD feed mice. After 12 weeks of treatment, total RNA was isolated from iIELs from control and HFD mice, each mRNA sample was quantified and the levels of mRNA of IL2, IL15, TNF α , IFN- γ , TGF- β , perforin and granzyme B were evaluated by conventional RT-PCR. The β -actin was used as internal control. (a) The PCR products were subjected to electrophoresis in 1.8% agarose gel, stained with ethidium bromide and visualized under UV light. (b) Quantitative real-time polymerase chain reactions (qRT-PCR) were performed using SYBR Green. RT-PCR results were normalized using β -actin as an internal control. Relative mRNA levels were calculated from the C_T values for each sample and the ratios were reported as arbitrary units (AU). * $p < 0.05$ compared with control mice by U Mann Whitney.

assessed by both conventional and semiquantitative RT-PCR. We found that HFD did not induce changes on mRNA levels of IL2, IL15 nor TGF- β (Fig. 5a and b). In contrast, mRNA levels of TNF- α and perforin were significantly higher in HFD mice compared to control (Fig. 5b). Interestingly, a huge mRNA level of granzyme B was detected in HFD mice, whereas a slightly granzyme B mRNA level was observed in iIELs from control mice (Fig. 5a and b). Although, high mRNA levels of IFN- γ were observed in both HFD and control mice, no significant differences were detected between both groups (Fig. 5a and b).

Discussion

Intraepithelial lymphocytes are localized into the intestinal wall. To date there is not enough information about the mechanisms how iIELs protect the local cells from antigens and

pathogenic micro-organisms; neither how cytokines produced by iIELs may regulate the local cellular functions and keep intestinal homeostasis.

It is well known, that consumption of a high fat diet (HFD) is associated with development of obesity in humans and animals. In mice, HFD induce disorders like those that occur in human, such as intestinal inflammation and insulin resistance.³² On the other hand, intestinal intraepithelial lymphocytes (iIELs) play an important role in the local immune response of the small intestine. iIELs are heterogeneous regarding to their phenotype, ontogeny, and functions. iIELs are organized in distinct subsets of T cells expressing either TCR $\gamma\delta^+$ or TCR $\alpha\beta^+$, with potent cytolytic and immunoregulatory functions. However, the mechanisms and functions of both TCR $\gamma\delta^+$ and TCR $\alpha\beta^+$ iIELs have not been completely elucidated. Herein, we have determined the effect of HFD induced obesity on iIELs subsets in



C57BL/6 mice. Our results clearly have shown that HFD induced a shift on proportion of TCR $\gamma\delta^+$ and TCR $\alpha\beta^+$ within the iIELs population.

A recent report described that HFD promotes an important decrease in the proportion of iIELs and goblet cells, altering the secretion of hormones as well as maintenance of intestinal barrier; but the TCR $\gamma\delta^+$ and TCR $\alpha\beta^+$ iIELs subsets were not studied.³⁴ Previous studies reported that different factors, such as pathogens and gut microbiota might change the proportions of iIELs. In this regard, infections with either *S. typhimurium*, *T. gondii*, or *N. brasiliensis* induced the expansion of iIELs expressing TCR $\gamma\delta$; suggesting that TCR $\gamma\delta$ contributed to maintain the integrity and function of intestinal epithelial tight junctions and goblet cells during infection.^{33,35–37} Similarly, beneficial interactions between commensal microbiota and iIELs expressing TCR $\gamma\delta$ have been described.^{38,39}

On the other hand, interactions between bacteria and HFD induced important changes in the gut microbiota¹⁰ and a proinflammatory response in the small intestine on progression of obesity was triggered.³² Therefore, it is clear that HFD induces changes on intestinal environment and iIELs might have an important role inducing local inflammation. However, so far there are few studies to elucidate the functions of iIELs in mice models of obesity.

In our experimental model of HFD induced obesity, the serum levels of cholesterol and glucose were similar between control and HFD mice; but an important increase on triglycerides and body weight was detected in HFD mice.

On this regards, different results on biochemical parameters have been published; some studies have shown that high fat diet increased blood levels of glucose, cholesterol, and triglycerides.^{40,41} Whereas de Sousa *et al.* have not observed changes on glucose and triglycerides, but an important increase on cholesterol was detected.⁴² Similarly, West *et al.* did not find any difference on glucose blood levels in several mice strains feed with HFD.⁴³ Discordance on these results could be due to different fatty acid profile on diet formulation,^{42,44,45} time of feeding mice,^{46,47} and mouse strain used.^{43,48} Thus, the effect of HFD induced obesity on iIELs was associated firstly with HFD induced obesity and not by other biochemical changes such as high levels of glucose (linked to type 2 diabetes mellitus) or cholesterol (linked to cardiovascular disease).

Moreover, an increase on proportion of TCR $\gamma\delta^+$ and a decrease on TCR $\alpha\beta^+$ within the iIELs population was found after 12 weeks of HFD. Previously, important changes in the proportions of bowel immune populations in the colon, including a reduced percentage of Tregs and an increase in IL-17-producing $\gamma\delta$ T cells, have been reported in mice feed with HFD.⁴⁹ A similar effect was reported using dietary nucleotides, and this phenomenon was dependent of IL-7 produced by intestinal epithelial cells.⁵⁰

iIELs modulate important biological cellular functions by their cytotoxic activity and cytokines production on the small intestine.²⁶ Here we have detected increased mRNA levels of TNF- α , perforin, and granzyme B in iIELs from HFD mice. In this regard, a recent report described that *in vivo* stimulation with anti-CD3 monoclonal antibody activate TCR $\gamma\delta$ to release granzyme B,

inducing DNA fragmentation on surrounding epithelial cells.⁵¹ In addition, a maximum cytotoxic activity of iIELs is preceded by perforin and granzyme B mRNA expression in iIELs after viral infection.⁵² Additionally, we showed higher mRNA levels of TNF- α in iIELs from HFD compared to control mice, suggesting that HFD promote the iIELs activation and synthesis of this cytokine. Consistently, previous reports have detected important changes on the levels of TNF- α mRNA after HFD feeding.^{34,53}

Interestingly, HFD did not induce any change on mRNA levels of TGF- β , IL2 and IL15; cytokines that promote proliferation and protection of intestinal barrier. Therefore, it is possible that HFD induced damage on intestinal barrier and deregulation on iIELs functions, increasing mRNA levels of TNF- α , perforin, and granzyme B. Previously, it has been described that HFD induced obesity is associated with alteration in occludin distribution and disruption of tight junctions of epithelial cells, a phenomenon that may result in an enhanced stimulation of iIELs and triggering an inflammatory event.¹¹ Curiously, high levels of IFN- γ mRNA were detected on iIELs of both groups, suggesting IFN- γ is not modified by HFD, suggesting that IFN- γ is mainly involved in specific immune response to intestinal damage induced by either bacteria or virus infection.^{54–56}

On the other hand, previous studies by DNA microarrays suggest that TCR $\gamma\delta^+$ iIELs could participate in the intestinal lipid metabolism and cholesterol homeostasis.⁵⁷ Thus, it is possible that increase of TCR $\gamma\delta^+$ cells by HFD could be induced by different diet components to maintenance of a stable equilibrium through physiological processes; whereas, TCR $\alpha\beta^+$ iIELs may have a more restrictive role on these functions. Therefore, it may reflect selective functions of TCR $\alpha\beta^+$ and TCR $\gamma\delta^+$ subsets on the intestine compartment of HFD mice. Although, the functional differences among TCR $\alpha\beta^+$ and TCR $\gamma\delta^+$ are still uncertain.

Conclusions

In summary, we have described that HFD induces important changes on proportion of iIELs as well as a significant increase on mRNA levels of TNF- α , perforin, and granzyme B in whole iIELs population. These findings may provide important insights into intestinal changes produced on HFD induced obesity, which could eventually trigger intestinal inflammation. However, further investigations are required to elucidate the specific molecular mechanism of HFD induced changes on iIELs and cytokines mRNA; primarily, because diet formulation (fatty acid profile) may produce different effect in gut.

Conflict of interest

All authors declare no conflict of interest.

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