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High Fat Diet-induced Obesity Enhances Allograft Rejection

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Abstract

Background—Obesity promotes a state of low-grade inflammation that exacerbates chronic inflammatory diseases such as asthma and inflammatory bowel disease. In transplantation, the survival of organs transplanted into obese patients is reduced compared to allografts in lean recipients. However, whether this is due to increased alloimmunity remains to be addressed conclusively.

Methods—We used a mouse model of high fat diet (HFD)-induced obesity and assessed immune responses to allogeneic stimulation *in vitro*, allogeneic splenocyte immunization *in vivo*, and allogeneic heart transplantation.

Results—Our results indicate that HFD altered the composition and phenotype of splenic antigen-presenting cells (APCs) that led to their enhanced capacity to stimulate T cells. Immunization with allogeneic splenocytes *in vivo* resulted in increased alloreactivity, as determined by IFN γ production. Moreover, cardiac allograft rejection in HFD mice was modestly accelerated compared to aged-matched control animals fed a low fat diet (LFD), correlating with enhanced alloreactive T cell function.

Conclusions—Our results highlight the increased alloresponse triggered by HFD-induced obesity and its negative impact on transplant outcome.

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INTRODUCTION

In the last 30 years, significant changes in diet and physical activity in the American people have greatly contributed to the current obesity epidemic¹. Obesity has a stark impact on public health, as it is associated with an increase in cardiovascular diseases, severity of asthma and gastrointestinal inflammatory diseases²⁻⁴. The estimated cost for direct obesity-related medical treatment in the US is over \$100 billion⁵ and this amount is increasing.

The location and duration of fat deposition impacts the severity of the disease, with visceral obesity being linked to inflammation of white adipose tissue⁶. At the cellular level, adipocytes produce inflammatory cytokines that enhance the prevalence of local pro-inflammatory M1 macrophages at the expense of anti-inflammatory and tissue-repairing M2 macrophages⁷. The change in the local cytokine milieu also impacts the local adaptive immune system, by recruiting CD4⁺ Th1 and CD8⁺ cytotoxic T cells while depleting Th2 and regulatory T (Tregs) cells involved in tissue homeostasis⁸. This local adipose inflammation may exacerbate chronic inflammatory diseases. Indeed, preclinical mouse models indicate that diet-induced inflammation increases the severity of T cell-mediated diseases in models of inflammatory bowel disease and multiple sclerosis⁹⁻¹¹.

The role for obesity in transplant alloreactivity remains controversial. When kidneys from a single donor were transplanted into different recipients, the organs grafted into obese patients displayed reduced long-term survival compared to the paired kidneys transplanted into lean recipients¹². However, whether this is due to increased alloimmunity or to non-immune factors leading to increased graft damage remains unclear as obesity is a risk factor for non-immune mediated comorbidities and surgical complications, which might affect graft function and fate^{3,13}. While some studies have reported an association between increased body mass index (BMI) with delayed graft function^{14,15} (itself a risk for acute rejection), and risk of acute rejection within the first 6 months post-transplantation^{15,16}, other studies have failed to reveal such associations^{17,18}. The discrepancy in the literature may be due to the fact that not all types of obesity seem to drive inflammation, as visceral obesity is more closely associated with inflammation¹⁹, and it may therefore be the type of obesity that impacts alloimmunity. Thus, despite elegant studies linking obesity to enhanced autoimmunity, there is a lack of consensus regarding whether obesity affects alloimmunity. In this study, we used a mouse model of HFD-induced obesity that has been reported to mimic inflammatory obesity linked with metabolic syndrome²⁰, to investigate the consequences of this type of obesity on alloimmune responses.

The composition of the immune system, as well as T cell responses were assessed in male C57BL/6 mice fed a HFD for at least 12 weeks, using age-matched control male mice fed a nutrient-equivalent LFD. APCs from mice fed a HFD elicited more robust proliferation and cytokine production by CD8⁺ and CD4⁺ T cells than APCs from LFD controls. HFD-fed mice displayed enhanced alloreactivity following immunization with fully allogeneic splenocytes. In a minor mismatched heart transplantation model, HFD mice rejected cardiac allografts with faster kinetics compared to their LFD counterparts, correlating with increased effector function of alloreactive T cells. Overall, our results indicate that HFD-induced

obesity exacerbates the alloimmune response, and suggest that obese patients could be at higher risk for T cell-mediated immune rejection of solid organ transplants.

MATERIALS AND METHODS

Mice and diets

Six-week old male C57BL/6 mice (Jackson Laboratories) were fed for at least 12 weeks a HFD or a LFD in which 60% or 10% kcal, respectively, came from fat (D12492i or D12450Bi, Research Diets Inc.). For most experiments, HFD diet weighed over 45 grams. Eight week-old female BALB/c and 129/Sv mice were purchased from Charles River and Taconic, respectively. Marilyn anti-male TCR transgenic female mice were obtained from NCI Frederickson and have been described previously²¹.

Weight and glucose assessment

Body weight was measured using a laboratory scale. Blood glucose was measured after 6h of fasting using glucose strips.

Purification of APCs and T cells

HFD and LFD mice were sacrificed, spleens removed, prepared into a single cell suspension and red blood cells lysed using ammonium chloride potassium hypotonic buffer. T cells were purified using the 'negative selection T cell purification' kit (StemCell) with magnetic beads. T cells were verified to have a purity of > 90% by flow cytometry and were labeled with 5 μ M CFSE. For preparation of splenic APCs, spleens were incubated for 10 min with biotin-labeled antibodies against CD4, CD8, Thy1.2 and NK1.1 (GK4.5, 53-6.7, Thy1.2 and NK1.1 from eBioscience), further incubated for 5 min with streptavidin-coupled magnetic beads (ThermoFisher) and finally negatively selected using a magnet (StemCell). The presence of T cells and NK cells was less than 1%. APCs were irradiated with 2000 rads using a Cesium-137 source. Cell quantification was performed using Countess (Invitrogen). 5×10^4 T cells were cultured in complete (c)DMEM (10% Fetal Calf Serum, 50 μ M β -mercaptoethanol, 1 $\times$ non-essential aminoacids, 1 $\times$ L-glutamine, 1 $\times$ penicillin/streptomycin and 10 mM HEPES) with 2×10^5 APCs in the presence of 1 μ g/ml of anti-CD3 monoclonal antibody (145-2C11 mAb) for 3 days at 37°C and 8% CO₂.

Flow cytometry

To assess the composition and phenotype of APCs, splenocytes ($1-2 \times 10^6$ cells/tube) resuspended in FACS buffer (1% BSA/PBS/ 0.09% sodium azide) were stained with fluorochrome-labeled antibodies against CD11b, CD11c, Gr-1, B220, K^b, IA^b, CD80, CD86, CD40 and fluorochrome-matched isotype controls. T cell preparations were stained with fluorochrome-labeled anti-CD4, -CD8, -CD44, -CD62L antibodies in FACS buffer, fixed and permeabilized in 'Foxp3 staining buffer' (eBioscience) and stained with anti-Foxp3 fluorochrome-labeled antibody. Antibodies were purchased from eBioscience and flow cytometry acquisition was performed on an LSR Fortessa (BD Biosciences). The analysis was performed using FlowJo software.

IFN γ ELISpot

IFN γ ELISpots were performed using 10^6 cervical lymph node or splenic cells from C57BL/6 mice, cultured for 18h in the presence of 2000 rads γ -irradiated T-depleted allogeneic splenocytes, using an IFN γ ELISpot kit and following the manufacturer's instructions (BD Biosciences). Spot quantification was performed using a C.T.L. quantification device.

Donor-specific transfusion (DST) immunization

C57BL/6 mice fed a LFD or HFD for >12 weeks were immunized subcutaneously with BALB/c splenocytes (10^6) at the base of the neck. Cervical lymph nodes draining this site were harvested 2 weeks later and their leukocytes were restimulated with syngeneic or donor irradiated splenocytes for analysis of IFN γ production by ELISpots, as described above.

Heart transplantation

Abdominal heterotopic cardiac transplantation was performed using a technique adapted from that originally described by Corry et al²². Briefly, cardiac allografts were transplanted in the abdominal cavity by anastomosing the aorta and pulmonary artery of the graft end-to-side to the recipient's aorta and vena cava, respectively. The day of rejection was defined as the last day of a detectable heartbeat.

Statistical analysis

Cardiac graft mean survival time, standard deviation and p-values were calculated using Kaplan–Meier/Log-rank test methods. Comparisons of means were performed using the Student's t-test, the Tukey test for multiple comparisons, or the Mann-Whitney test when appropriate.

RESULTS

HFD-induced obesity enhances the stimulatory capacity of APCs *in vitro*

As a first step to test the impact of HFD-induced obesity on alloimmunity, aged-matched male C57BL/6 mice were fed for at least 12 weeks a HFD (60% kcal from fat) or a control LFD (10% kcal from fat). As expected, the body weight and fasting blood glucose levels were increased in HFD-fed when compared to LFD-fed mice (Fig. 1).

To determine if HFD-induced obesity affected the systemic T cell compartment, the composition of T cells was assessed by flow cytometry in splenocytes from HFD and LFD mice. No differences were observed in the frequencies (Fig. 2A) or total numbers (not shown) of CD4⁺, CD8⁺, CD4⁺Foxp3⁺ Tregs, or CD44^{lo}CD62L^{hi} naïve and CD44^{hi}CD62L^{lo} memory subsets. To assess whether HFD modulates T cell responses, CFSE-labeled T cells from HFD or LFD mice were stimulated for 3 days with equal numbers of APCs (T- and NK-depleted splenocytes) from HFD or LFD mice in the presence of activating soluble anti-CD3 mAb. Strikingly, anti-CD3 mAb induced greater proliferation of CD4⁺ and CD8⁺ T cells when in the presence of HFD compared to LFD APCs, regardless of whether T cells originated from HFD or LFD mice (Fig. 2B). Similarly, IL-17 expression by CD4⁺ and IFN γ

production by CD8⁺ T cells were increased when T cells were stimulated with HFD, as compared with LFD, APCs, irrespective of the T cell provenance (Fig. 2C).

To investigate if these effects held true during cognate alloreactive T cell interactions and to exclude the possibility that differences were due to a different TCR repertoire in HFD versus LFD mice, we investigated the proliferative capacity of TCR-transgenic T cells from lean Marilyn female mice, in which monoclonal CD4⁺ T cells recognize an H-Y peptide presented by I-A^b. CFSE-labeled Marilyn T cells were stimulated with APCs from HFD or LFD male mice, and similarly to the polyclonal T cell response, the proliferation of Marilyn T cells was greater when cultured with HFD than LFD APCs (Fig. 2D). These results indicate that APCs from HFD mice have an increased T cell stimulatory capacity when compared to APCs from LFD mice, suggesting that the APC compartment in HFD mice was altered, either in composition or in phenotype.

HFD-induced obesity alters the composition and phenotype of splenic APCs

To investigate whether the APC composition in the spleen was altered by the HFD, the percentages and total numbers of macrophages (CD11b⁺CD11c^{-int}Gr1⁻), conventional dendritic cells (cDC, CD11c⁺CD11b⁺), plasmacytoid DCs (pDCs, CD11c^{int}B220⁺CD11b⁻), CD8α DCs (CD11c⁺CD8α⁺CD11b⁻), B cells (B220⁺CD11c⁻CD11b⁻) and granulocytes (FSC^{hi}SSC^{hi} Gr1⁺) were analyzed by flow cytometry. The percentages and total numbers of cDCs, macrophages and granulocytes were increased in splenocytes from HFD compared to LFD mice (Fig. 3A, B). Importantly, cellularity of the spleen from HFD mice was greater than that from LFD mice, such that the total numbers of B cells and pDCs were also significantly greater in splenocytes from HFD than LFD mice (Fig. 3B). In addition, macrophages and cDCs from HFD mice expressed higher levels of the costimulatory molecule CD80 than their counterparts from LFD mice (Fig 3C), providing a possible explanation for the greater activation ability of HFD APCs.

HFD-induced obesity results in greater alloresponse to allogeneic DST immunization

Our *in vitro* results support the hypothesis that HFD mice may develop stronger T cell immune responses *in vivo*. To test this possibility, LFD or HFD C57BL/6 mice (H-2^b) were injected subcutaneously in the base of the neck with splenocytes from BALB/c mice (H-2^d). Cells from the draining lymph nodes were harvested 2 weeks later and restimulated with syngeneic (C57BL/6) or donor (BALB/c) irradiated splenocytes to determine the frequency of IFNγ-producing cells. Unimmunized naïve C57BL/6 mice fed normal chow were used as controls. As shown in Figure 4, the production of IFNγ by cells from BALB/c-immunized HFD mice was significantly greater than that from BALB/c-immunized LFD mice. Thus, HFD-induced obese mice displayed increased alloreactivity to DST *in vivo*.

HFD-induced obesity results in faster cardiac allograft rejection kinetics

To determine the effect of HFD on the alloimmune response towards solid organs, hearts from BALB/c females were transplanted into C57BL/6 HFD or LFD male mice. In this fully mismatched strain combination, both LFD and HFD mice rejected their grafts within 7–8 days (data not shown). Because the tempo of rejection of fully mismatched cardiac allografts may already be maximal in LFD mice, we investigated the effect of HFD-induced obesity on

the kinetics of rejection of minor mismatched allografts. To this end, hearts from 129/Sv (H-2^b) females were transplanted into C57BL/6 (H-2^b) HFD and LFD male mice. In this strain combination, cardiac allografts transplanted into HFD recipients underwent an accelerated rejection compared to LFD recipients, whereas obesity did not affect the survival of syngeneic heart transplants (Fig. 5A). Allospecific T cell responses, measured 2–3 weeks post-rejection, were significantly increased in transplanted HFD mice, as demonstrated by higher 129/Sv-specific IFN γ ELISpots (Fig. 5B). This difference was not solely driven by the one high producer, as elimination the highest IFN γ -producer in each group still resulted in statistically significant differences (data not shown). Taken together, our data support the conclusion that HFD-induced obesity moderately exacerbates alloimmune responses and can accelerate rejection of solid organs.

DISCUSSION

Most studies suggest that obesity has a negative impact on graft outcome^{23–28}. However, because obesity is associated with surgical complications, cardiovascular comorbidities, and other factors that may negatively impact transplant function and half-life, whether obesity directly affects alloimmunity is unclear. Using an experimental setting that has been reported to model inflammatory obesity, we tested directly whether HFD-induced obesity impacts T cell responses and alloimmunity. Our results indicate that HFD-induced obesity modestly enhances T cell alloimmunity and accelerates allograft rejection.

Several animal models of obesity have been used in various experimental settings, including genetic- and diet-induced models. The genetic models comprise mice that are deficient in the leptin/leptin receptor axis and become obese from hyperphagia²⁹, whereas the HFD-mediated obese mice have increased leptin levels (data not shown) and do not display hyperphagia but rather have calorie-rich diet-induced weight gain. We used male C57BL/6 mice subjected to a HFD because they have been reported to develop obesity, hyperinsulinemia, hyperglycemia and hypertension that mimic the metabolic syndrome observed in patients with inflammatory obesity, whereas other mouse strains are more resistant to these effects²⁰. In addition, we used a LFD as a control diet, as opposed to normal chow, to ensure that only the kilocalories from fat differed between the 2 diets such that possible differences in graft outcome could not be ascribed to different dietary antigens between the 2 feeds. Our results using HFD-fed obese male C57BL/6 mice demonstrate that HFD leads to increased T cell proliferation, or IFN γ production in response to alloantigen in 3 independent models: *in vitro* using Marilyn T cells stimulated with minor antigen-expressing APCs, *in vivo* following DST immunization with fully allogeneic splenocytes, and *in vivo* in a model of minor mismatched heart transplantation.

The rejection kinetics of heart allografts in HFD obese mice were only modestly accelerated compared to LFD mice, even when all parameters were carefully controlled: LFD and HFD hosts genetically identical and aged-matched, matched composition of the LFD and HFD that differed only in kilocalories from fat, controlled environment. Moreover, the effects of HFD-induced obesity were only detectable in mice transplanted with minor rather than major mismatched cardiac allografts and when HFD had been administered for over 12 weeks but not 8–10 weeks (data not shown). These data suggest that it takes time for the

immune consequences of obesity to take hold. Similarly, frequency of IFN γ -producing cells following *ex vivo* donor restimulation was only slightly increased in HFD versus LFD mice. This may be due to the fact IFN γ production was measured 2–3 weeks post-rejection. At this time point, the differentiation of alloreactive T cells into effector/memory cells in LFD mice may have caught up with that in HFD mice. Future experiments investigating the kinetics of effector alloreactive T cell differentiation following transplantation should be able to determine if differences between LFD and HFD mice become more apparent when compared at a time point just before rejection starts occurring in the HFD mice. The modest effects of HFD-induced obesity may explain the difficulty in detecting the consequences of obesity on alloimmunity in obese patients with different degrees of genetic disparities between donors and recipients, different duration of obesity, and when all types of obesity are considered together. In patients, it has been suggested that waist circumference or waist-to-hip-ratio may be a better prognostic factor for transplant outcomes than BMI^{19,30,31}, as abdominal fat rather than fat accumulation in other parts of the body has been linked with increased inflammation³² and therefore may be the type of obesity that results in enhanced alloimmunity.

The fact that not all types of obesity may increase alloimmunity is exemplified by results in leptin-deficient obese mice that were transplanted with allogeneic skin grafts and displayed prolongation of graft survival rather than accelerated graft rejection³³. In this case, leptin deficiency was associated with increased Tregs and a Th2 profile, prompting the authors to speculate that the adverse outcome of transplanted organs in obese patients may be due to increased leptin levels, which are pro-inflammatory. Because HFD-induced obesity in male C57BL/6 mice but not leptin-deficiency is associated with visceral inflammation and because HFD C57BL/6 mice display elevated levels of serum leptin (data not shown), our results are consistent with this interpretation and we favor the hypothesis that only inflammatory obesity leads to enhanced alloimmunity. However, serum levels of TNF α , IL-1 β and IL-6 were similar in our lean and HFD mice (data not shown), suggesting that visceral inflammation may be sufficient to promote alloreactivity in obesity without requiring detectable levels of systemic inflammation in the circulation. Alternatively, visceral inflammation and increased alloreactivity may be 2 independent consequences of obesity.

Our results suggest that the basis for increased alloreactivity may be due to APCs from HFD mice having intrinsically enhanced capacity to stimulate T cells, correlating with increased expression of the costimulatory molecule CD80. These results are consistent with a recent report showing that both aging mice that acquire increased visceral adiposity as they get older, and HFD-fed obese mice respond with higher cytokine production than lean mice to immune stimulation *in vivo* with anti-CD40 and IL-2³⁴. Our results also show increased production of IL-17 by CD4⁺ T cells stimulated with APCs from HFD mice, consistent with data showing that obesity leads to a Th17 bias and increased susceptibility to autoimmunity³⁵. The mechanisms that link obesity to increased baseline APC function and increased Th17 frequency as well as subsequent adaptive alloreactivity remain to be elucidated.

Overfeeding has been linked with increased intestinal permeability to endotoxin³⁶, which may cause innate inflammation and be able to lead to secondary augmentation of adaptive alloimmunity. Moreover, diet is a strong determinant of the microbial composition of the intestine^{37,38}. Because there is a known cross-talk between the microbiota and the immune system^{39,40}, one possibility is that obesity-induced dysbiosis affects systemic alloimmunity⁴¹. Another possibility is that obesity can be also linked to hyperlipidemia, and hyperlipidemia has recently been shown to promote accelerated rejection of cardiac allografts in mice, by inducing anti-donor Th17 reactivity⁴². Similarly, obesity is often associated with hyperglycemia and our HFD animals displayed higher serum levels of fasting glucose. However, whether hyperglycemia is another variable that affects alloimmunity remains to be determined. Obesity is also linked to an increased ratio of the pro-inflammatory adipokine leptin to the anti-inflammatory adipokine adiponectin, which may skew immune responses towards increased alloreactivity and accelerated acute rejection. Indeed, as previously mentioned, mice deficient in leptin had prolonged skin allograft survival and enhanced Treg and Th2 responses³³. In contrast, mice deficient in adiponectin displayed more rapid cardiac allograft rejection⁴³. Additional factors have linked obesity to inflammation, including inflammatory lipids⁴⁴. Interestingly, atorvastatin, an inhibitor of HMG-CoA reductase that reduces levels of cholesterol, was described to have immunomodulatory capacity in models of cardiac transplantation⁴⁵ and a retrospective analysis in the same study revealed that kidney transplant patients taking statins had an increased overall survival compared to the non-statin treated group. In addition to pharmacological treatment, obesity can be reduced with lifestyle changes or bariatric surgery. It would be of interest that future studies test whether lifestyle and surgical treatment of obesity restore a lower T cell activation capacity by APCs and normalize kinetics of transplant rejection.

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Abbreviations

APC	antigen-presenting cell
BMI	body mass index
DST	donor-specific transfusion
HFD	high fat diet
LFD	low fat diet
MST	mean survival time
Treg	Regulatory T cell

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Figure 1. Mice fed a HFD are obese and hyperglycemic

Weight (A) and fasting glucose levels (B) of C57BL/6 male mice (mean $\pm$ SEM) fed for >12 weeks a HFD (n=6) or a LFD (n=6). Comparisons were performed using a two-tailed unpaired t test. **: $p < 0.01$, ****: $p < 0.0001$.

Figure 2. HFD results in greater T cell activation

Percentages of CD4⁺, CD8⁺, Tregs, memory and naïve T cells (A) in splenocytes from HFD and LFD mice were analyzed by flow cytometry. Proliferation (B), IFN γ production by CD8⁺ cells (C) and IL-17 expression by CD4⁺ cells were assessed in HFD and LFD T cells stimulated with HFD or LFD APCs. (D) Proliferation of CFSE-labeled CD4⁺ Marilyn T cells stimulated for 3 days with APCs isolated from mice maintained on HFD or LFD for >12 weeks. Experiments were performed in groups of at least 3 mice and repeated at least twice. Results are displayed as mean $\pm$ SEM. Comparisons were performed using a two-tailed unpaired t test. **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

Figure 3. HFD alters the composition and stimulatory capacity of splenic APCs

APCs were isolated from the spleens of mice maintained on HFD or LFD for >12 weeks. Percentages (A) and total numbers (B) of splenic macrophages (CD11b⁺CD11c^{-int}Gr1⁻), conventional DCs (cDC, CD11c⁺CD11b⁺), plasmacytoid DCs (pDC, CD11c^{int}B220⁺CD11b⁻), CD8 α DCs (CD8-like DC, CD11c⁺CD8 α ⁺CD11b⁻), B cells (B220⁺CD11c⁻CD11b⁻) and granulocytes (FSC^{hi}SSC^{hi} Gr1⁺) were analyzed by flow cytometry. (C) MFI analysis of CD80 expression on macrophages and cDCs, as assessed by flow cytometry. Dotted lines represent MFI for isotype control. Three animals were analyzed

in each group and the experiment was repeated twice. Results are displayed as mean $\pm$ SEM. Comparisons were performed using two-tailed unpaired t test. *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$, ns: not significant.

Figure 4. Mice in HFD develop increased alloreactivity to *in vivo* DST immunization
 C57BL/6 mice (H-2^b) in LFD and HFD were immunized with allogeneic BALB/c splenocytes (10⁶ cells) subcutaneously under the skin of the neck and cells isolated from cervical draining lymph nodes were analyzed 14 days later by ELISpot for their IFN γ production upon *in vitro* restimulation with irradiated syngeneic or donor splenocytes. The data (mean $\pm$ SEM of 3 mice/group) are representative of 2 independent experiments with at least 3 mice/group. Comparisons were performed by one-way ANOVA. *: $p < 0.05$.

A)

B)

Figure 5. Mice in HFD have faster kinetics of cardiac allograft rejection

(A) Rejection kinetics of syngeneic (C57BL/6, LFD n=5, HFD n=5; both survived >d30 post-transplantation), and minor mismatched 129/Sv (H2) cardiac allografts (LFD n=5, mean survival time $\pm$ SD = 17 $\pm$ 5; HFD n=6, mean survival time $\pm$ SD = 10 $\pm$ 3) C57BL/6 mice. Comparisons were performed using Kaplan–Meier/Log-rank test. (B) Splenocytes were harvested from all the mice in A, 2–3 weeks following rejection of 129/Sv cardiac allografts by LFD and HFD C57BL/6 recipients. An IFN γ ELISpot assay was performed upon overnight restimulation with γ -irradiated T cell-depleted 129/Sv

splenocytes. Data are displayed as mean $\pm$ SEM. Comparisons were performed using a one-tailed Mann-Whitney test.

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