Obesity accelerates thymic aging

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Abstract

As the expanding obese population will grow older, their successful immunological aging will be critical to enhancing the health-span. Obesity increases risk of infections and cancer, suggesting adverse effects on immune-surveillance. Here we report that obesity compromises the mechanisms regulating T cell generation via inducing premature thymic involution. Diet-induced obesity (DIO) reduced thymocyte counts and significantly increased apoptosis of developing T cell populations. Obesity accelerated the age-related reduction of T cell receptor excision circle (TREC) bearing peripheral lymphocytes, an index of recently generated T cells from thymus. Consistent with reduced thymopoiesis, dietary obesity led to reduction in peripheral naïve T cells with increased frequency of effector-memory cells. Defects in thymopoiesis in obese mice were related with decrease in the lymphoid primed multipotent progenitor (MPP:Lin−Sca1+Kit+ Flt3+) as well as common lymphoid progenitor (CLP:Lin−Sca1+CD117loCD127+) pool. The T cell receptor (TCR) spectratyping analysis revealed that obesity compromised V-β TCR repertoire diversity. Furthermore, the obesity induced via melanocortin 4 receptor deficiency also constricted the T cell repertoire diversity, recapitulating the thymic defects observed with diet-induced obesity. In middle-aged humans, progressive adiposity with or without Type-2-Diabetes also compromised thymic output. Collectively, these findings establish that obesity constricts T cell diversity by accelerating age-related thymic involution.
Introduction

It is well recognized that induction of negative energy balance by calorie restriction without malnutrition robustly enhances mean and maximal life-span \(^1\). The reduced calorie consumption in primates and mice also forestalls the aging of thymus and prevents immunosenescence \(^2,^3\). In contrast, obesity, associated with caloric excess increases risk of multiple comorbidities that adversely affect the health and life expectancy \(^4\). Although, excessive calories and obesity reduces health-span, it is unclear whether this involves the mechanisms governing the generation of T cells in thymus \(^4\).

The process of thymic aging is characterized by reduced production of naïve T cells and replacement of lympho-stromal thymic zones with adipose tissue \(^3-^7\). The reduction of thymus derived naïve T cells with age and homeostatic expansion of memory T cells restricts the T cell repertoire diversity and leads to immunosenescence \(^8\). Consequently, the process of age-related thymic involution contributes to increased susceptibility to infections, cancer and higher risk of vaccination failures in elderly \(^8-^11\).

Obesity is a multisystem disorder associated with aberrant neuroendocrine response to chronic calorie excess. In US alone, obesity is responsible for approximately 300,000 deaths per year \(^12^-^14\). The type 2 diabetes, cardiovascular disease (CVD) and cancers constitute majority of obesity-related mortality and morbidity \(^14\). However, the non –CVD and non-cancer deaths, due to chronic and acute infections also contribute to substantial adult mortality in obese individuals \(^14\). Furthermore, obese subjects are susceptible to post-operative and nosocomial infections and more likely to develop serious complications from common infections \(^4,^15\). In addition, obesity compromises the innate immune responses to the bacterium *Porphyromonas gingivalis* \(^16\). The obese mice infected with *P gingivalis* display increased periodontal pathology and a blunted
expression of pro-inflammatory cytokines \(^{16}\). Therefore, a non-specific low grade ‘sterile’ chronic inflammation seen during obesity \(^{17}\) is in some respects similar to age-related inflammation \(^{18}\) and does not impart advantage to the host with regards to mounting a specific pro-inflammatory response against specific pathogens. Consistent with these data, obese dogs have a greater susceptibility and increased mortality to canine distemper virus infection \(^{19}\). In addition to reduced vaccination response \(^{20, 21}\) obesity also leads to 6 fold increase in mortality post influenza infection \(^{22}\) with impaired antigen-specific CD8 T cell responses \(^{23}\).

The unique three-dimensional thymic structure is comprised of the cortex and the medulla which are composed mainly of distinct developing T cell subsets and thymic stromal cells \(^{24-26}\). The cortical and medullary thymic stromal cells provide a unique environment, cell-cell contact and produce growth factors required for various aspects of T cell development \(^{24, 26}\). The cortical thymic epithelial cells (cTECs) regulate the migration and expansion of T cell progenitors including the positive and negative selection of developing thymocytes \(^{27}\). The medullary TECs along with antigen presenting dendritic cells are responsible for deletion of self-reactive T cells and support the late stages of T cell development \(^{27-29}\). The thymopoietic potential is compromised with increasing age due to multiple causes including loss of TEC populations \(^{3, 30}\), defects in hematopoietic stem cells (HSCs) and reduction in earliest thymocyte progenitors (ETPs) \(^{31, 32}\) and alteration in growth factors and hormones \(^{33}\).

Whether obesity and prolonged nutrient excess impact the mechanisms of thymic involution process remains to be determined. However, previous studies examining immune function in extreme monogenic rodent models of obesity \((\text{Lep}^{ob}/\text{Lep}^{ob})\) have demonstrated clear thymic involution \(^{34}\) and significant defects in T
cell responsiveness. However, leptin directly affects T cells via functional leptin-receptors and can promote thymic function independently of obesity. The effects of diet and hyperphagia versus direct leptin signaling on immune system have not been delineated and it is not certain whether obesity alone accounts for thymic deficits in \textit{Lep} \textit{ob}/\textit{Lep} \textit{ob} mouse model. Furthermore, the loss of function leptin mutations in humans account for a minute fraction of current diet induced obesity “epidemic”.

Whether obesity induced by hyperphagia affects thymic function has not been examined. Among the genetic causes of obesity, loss of function mutation in Melanocortin 4-receptor (\textit{Mc4r}) remains the most common form of monogenic human obesity accounting for approximately 4-6\% obesity prevalence. The activation of \textit{Mc4r} in the hypothalamus and brain stem initiates an anorexigenic response, and also stimulates energy expenditure through the sympathetic nervous system. Ablation of \textit{Mc4r} gene in mice results in hyperphagia and obesity which is associated with insulin resistance, but not frank type 2 diabetes in mice fed standard chow diet.

We tested the hypothesis that obesity induced by high fat diet or chronic hyperphagia would accelerate age-related loss of thymic function. We therefore used \textit{ad libitum} high-fat diet feeding to induce dietary obesity and tested the impact of resultant adiposity. To test whether chronic hyperphagia (upon \textit{ad libitum} intake of chow diets) induced caloric excess would recapitulate the high-fat diet induced obesity, we ablated the \textit{Mc4r} mediated satiety signals. The \textit{Mc4r} knock out mouse model also allows the separation of defects induced by excess caloric intake versus direct effects of \textit{Mc4R} signaling on lymphoid system as \textit{Mc4R} is restricted to CNS. We provide evidence that adiposity compromises the mechanisms regulating thymic function. This involves reduction in T cell progenitors, increased apoptosis of thymocyte subsets and
lower thymopoiesis. We also demonstrate that compared to lean and overweight humans, development of obesity with and without type-2-diabetes (T2D) significantly reduces the number of recent thymic emigrants.
Materials and methods

Material and reagents.
For FACS analysis following antibodies (from eBiosciences Inc) were used, CD4-PerCP, CD8-APC, CD44-FITC, and CD4-PE, CD4-FITC, CD4-PerCP, CD11b, Gr-1-PE, CD45R-PE, CD3-PE, CD8-PE, αβTCR-PE, γδTCR-PE, pan-NK-PE, NK1.1-PE, CD11c-PE, CD19-PE, Ter119-PE and CD127-PE and CD127-APC, Flt3-FITC, CD25-APC and C-kit-FITC, Sca1-PE.

Subject population.
The TREC assay was performed on subjects undergoing clinical testing at baseline from various clinical trials at the Pennington Biomedical Research Center (PBRC-Cohort). The buffy coat cells from lean, overweight, obese and morbidly obese subjects were frozen and archived. All analyses were performed under an approved Pennington Biomedical Research Center IRB protocol and informed consent was obtained in accordance with the Declaration of Helsinki. The Type 2 diabetes was defined based on measurement of fasting serum glucose and insulin levels as described before 12-14.

Mice and animal care
Male C57BL/6 mice were fed ad libitum high-fat diet consisting of 60% calories from fat (D12492i; Research Diets Inc) starting at 8 weeks of age and control male mice were fed a standard chow diet consisting of 4.5% fat (5002; LabDiet). The Mc4r−/− have been described previously 41-44. The Mc4r−/− and Mc4r+/+ mice were provided with ad libitum chow diet. The animals were individually housed in specific pathogen free barrier facility with a 12-hour light/12-hour dark cycle with free access to food and water. All
animal use was in compliance with the Institute of Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Use and Care Committee at Pennington Biomedical Research Center.

**Isolation of organs and cell suspension**

After sacrifice, thymi and spleen were isolated and weighted. They were fixed with 20% sucrose solution and frozen for cryosectioning or RNA extraction, and dispersed on nylon mesh for single-cell preparation. The dispersed cells were treated with ACK lysing buffer (Quality biological, Inc) and prepared as single-cell suspensions purification of CD4 cells using negative selection and magnetic bead based methods. The Meyer hematoxylin and eosin (Dako) staining was performed on frozen thymic sections as described previously 33.

**FACS analysis**

Thymic lobes were minced into small fragments and treated 3 times for 10 min at 37°C with an enzymatic mixture containing 0.125% collagenase D (Roche Diagnostics), 0.1% DNase type I (Roche Diagnostics) in RPMI 1640. The remained thymic fragments were treated with 0.125% collagenase/dispase (Roche diagnostics), 0.1% DNase I in RPMI 1640. The collected cells were filtered through 100-μm mesh and spun at 1,300 rpm for 5 min and used for cell counting and staining. Immunofluorescent staining was performed as previously reported 33. The ETPs 31,32 were identified as described previously, 5 x 10⁶ thymocytes were labeled for lineage positive cells by utilizing PE-conjugated anti-CD11b, Gr-1, CD45R, CD3, CD8, αβTCR, γδTCR, pan-NK, NK1.1, CD11c, CD19, Ter119 and CD127 antibodies but no CD4, followed by staining with
CD25-APC and C-Kit-FITC. The apoptosis was determined using by Annexin V staining kit per manufacturer’s instructions. The MPP cells were stained as Lin-Sca1⁻Kit⁺ Flt3⁺, CLP as Lin⁻Sca1⁺Kit⁺CD127⁺. The Lin⁻Sca1⁻Kit⁺⁻CD127⁻ myeloid progenitors were further stained with CD34 and CD16CD32, to resolve common myeloid progenitor (CMP; CD34⁺CD16CD32⁻), granulocyte-monocyte progenitors (GMP; CD34⁺CD16CD32⁻), and megakaryocyte-erythrocyte progenitors (MEP; CD34⁻CD16CD32⁻). The splenocytes were stained with CD4-APC, CD8-PercP and CD62L-FITC and CD44-PE to identify the naïve (CD62L⁺CD44⁻ and memory CD62L⁻CD44⁺). All the FACS analysis was performed on a FACSCalibur (BD Biosciences) using up to four fluorescent channels and all FACS data was analyzed by post collection compensation using FlowJO (Treestar Inc) software.

**Real-time RT-PCR and signal joint-TREC analysis**

The total RNA was prepared with RNAzol (Isotex Diagnostics). The cDNA synthesis and real-time RT-PCR was performed as described previously (BioRad) ³, ³³. Real-time RT-PCR analyses were done in duplicate on the ABI PRISM 7900 Sequence Detector TaqMan system with the SYBR Green PCR kit as instructed by the manufacturer (Applied Biosystems). The list of real-time PCR primers is shown in supplementary table 1. The GAPDH or β-actin was used a housekeeping gene or internal standard for normalization. The Ct values of quantitative real time PCR were calculated using a standard \( \Delta \Delta Ct = \Delta Ct \) (control) - \( \Delta Ct \) (test). A \( \Delta Ct \) value is calculated for each sample as the difference between the Ct values for the gene of interest and the housekeeping gene in each sample.
The CD4+ T subsets were isolated from splenocytes using mouse CD4 T cells positive selection kit (Invitrogen). The sorted cells were lysed in 100 mg/L proteinase K (Sigma) for 1 h at 56°C followed by 10 min at 95°C. The amount of TREC in 5 x 10^6 cells was determined by real-time quantitative PCR using the ABI PRISM 7900 Sequence Detector TaqMan system (Applied Biosystems). The PCR performed with \( m_\delta \)Rec and \( \psi J_\alpha \) specific primers and \( m_\delta \)Rec-\( \psi J_\alpha \) fluorescent probe as described previously. The standard curves for murine TREC were generated by using \( \delta \)Rec \( \psi J_\alpha \) TREC PCR product cloned into a pCR-XL-TOPO plasmid, a generous gift from Dr. Gregory D. Sempowski, Duke University Medical Center. The human TREC analysis was performed on frozen PBMC samples derived from PBRC-cohort buffy coat samples in the clinical data base repository. The human TREC plasmid was a generous gift from Dr. Daniel Douek, National Cancer Institute NIH.

**V\( \beta \) TCR spectratyping analysis**

The CD4 T cells were used to prepare total RNA and cDNA was prepared as described previously. A FAM-labeled nested constant \( \beta \)-region primer was used in combination with 24 multiplexed forward murine V\( \beta \)-specific primers to measure the CDR3 lengths as described previously. Each peak was analyzed and quantified with ABI PRISM GeneScan analysis software (Applied Biosystems), based on size and density. Data were used to calculate the area under the curve (AUC) for each V\( \beta \) family. Each peak, representing a distinct CDR3 of a certain length, was quantified with BioMed Immunotech software.

**Statistical Analyses**
The results are expressed as the mean ± SEM. The differences between means and the effects of treatments were determined by one-way ANOVA using Tukey’s test (Sigma Stat), which protects the significance ($p < 0.05$) of all pair combinations.
Results

Diet-induced obesity is associated with premature thymic involution

In mice, the involution of thymus and reduction in T cell receptor excision circles (TRECs) as a measure of recent thymic emigrants is detectable within a year of age. To investigate whether obesity affects thymic involution, at 6 weeks of age, the control mice were placed on chow diet and DIO mice were given high-fat diet (60% kcal fat). The control and DIO male mice fed ad libitum chow and high-fat diets were aged for 13 months. At one year of age, C57BL/6 mice fed the high-fat diet were significantly heavier compared to chow fed controls (Figure 1A). A gross examination of thymi from 13m old DIO mice revealed marked increase in perithymic adipose tissue (PTAT) and thymus appears to morph into PTAT of obese mice (Figure 1A and 1B). Compared to chow fed control lean animals, the DIO animals had higher body weights and thymi had significant reduction in total thymocyte counts (Figure 1A). Histological examination of thymi from DIO mice revealed reduced cortical and medullary cellularity and obliteration of cortico-medullary junctions (Figure 1B). Analysis of thymocyte subsets revealed that DIO mice had reduced frequency and numbers of single positive (SP) CD4+, CD8+ and CD4+CD8+ double positive (DP) cells (Figure 1C). In addition, compared to lean animals, obesity significantly increases the apoptosis of thymocyte subsets (Figure 1D). These findings suggest that obesity associated with consumption of a calorie-dense high fat diet increases age-related thymic involution.

Progressive obesity inhibits thymopoiesis and restricts T cell repertoire diversity

Reduction in the ability of thymus to produce naïve T cells is reflected by decrease in peripheral CD62L+CD44- naïve cells. To determine whether dietary-obesity regulates thymic output, we next aged the mice fed a high fat diet and control chow diet and
analyzed the CD4 and CD8 naïve and effector/memory (E/M) T cells in spleen at 3, 9 and 13 months of age. We found that at 3m, dietary-obesity did not affect the naïve and memory T cell frequency (Figure 2). Interestingly, the age-related decline in naïve CD4 and CD8 cells and homeostatic expansion of E/M cells was significantly accelerated with development and duration of dietary-obesity and adiposity (Figure 2).

Because the presence of naïve recent thymic emigrants (RTEs) cannot yet be distinguished by specific surface markers, the quantitation of extrachromosomal DNA circles, termed TCR rearrangement excision circles (TRECs), has been used to evaluate thymic output \(^{45,33}\). Consistent with our findings that obesity accelerates age-related deterioration of thymic architecture, reduces thymocyte counts with increased thymocyte subset apoptosis, the TREC numbers in obese mice were significantly lower than age-matched chow fed leaner controls (Figure 3A). Furthermore, compared to lean controls, the T cells isolated from 13mo old DIO mice expressed significantly reduced expression of key cytokines, IFN\(\gamma\), TNF\(\alpha\), IL-6 and TGF-\(\beta\)1 without affecting IL-2 and IL-4 mRNA levels (Figure 3B). These data suggest that steady-state mRNA expression of pro-inflammatory cytokine expression in splenic T cells in obese mice is lower and is consistent with recent report suggesting that upon influenza infection the CD8 T cells have deficient IFN\(\gamma\) production and response \(^{22,23}\).

The reduction in naïve T cell output and expansion of memory T cells due to diminished thymopoiesis results in reduced TCR diversity and consequent functional deficits in adaptive immune responses \(^{8,48,49}\). We therefore next studied the TCR diversity of peripheral CD4\(^+\) T cells by measuring the distribution of lengths of the complementarity determining region 3 (CDR3), which though a lower resolution approach, allows a global analysis of the TCRs of the sampled T cell population \(^{46,47}\). A
polyclonal diverse TCR Vβ family is characterized by Gaussian distribution of peaks, while a skewed profile is distinguished by deviations from Gaussian distribution and aberrant amplification of peaks. The Gaussian distribution profiles were translated into probability distributions as functions of the area under the curve for each CDR3 length as described previously. By these methods we demonstrate quantitatively that the progressive dietary-obesity restricted TCR diversity with little effect of high-fat diet-induced obesity in 3mo old animals (Figure 3C).

**Dietary Obesity reduces lymphoid and increases myeloid progenitors**

T cell development in the thymus is dependent on periodic seeding of lymphoid progenitors from bone marrow. The multipotent self-renewing hematopoietic stem cells (HSC; Lin’Sca1’kit’Flt3’ or Flt3 LSKs) in bone marrow differentiate into non-renewing population of lymphoid primed multipotent progenitor (MPPs; Flt3’LSKs). The MPPs further give rise to a more differentiated common lymphoid progenitor (CLP; Lin’Sca1’KitloCD127+). Given, dietary-obesity increases thymic involution we tested whether the obesity reduces the pool of MPP and CLP progenitor populations. We found that obesity did not alter the LSK number but compared to 13mo old lean controls it significantly reduced the frequency of MPP and CLP populations (Figure 4A) suggesting that obesity decreases lymphoid progenitors in BM.

Considering that LSK can generate multiple hematopoietic lineages, we next sought to determine whether obesity-induced shrinkage of MPP and CLP pool also impacted the non-lymphoid cell lineages. The myeloid progenitors are known to exist within the Lin’Sca1’KithiCD127+ fraction of bone marrow cells. On the basis of expression of CD34 and CD16CD32, the Lin’Sca1’KithiCD127+ myeloid progenitors can be further resolved into common myeloid progenitor (CMP; CD34+CD16CD32lo),
granulocyte-monocyte progenitors (GMP; CD34⁺CD16CD32⁺), and megakaryocyte-erythrocyte progenitors (MEP; CD34⁻CD16CD32⁻). Dietary obesity significantly (p<0.05) increases the frequency of CMP, MEP while reducing the GMP cells in BM (Figure 4B). These data suggest that obesity does not lead to a general defect in HSCs that non-specifically reduces multiple hematopoietic lineages. Rather obesity selectively compromises the lymphoid progenitor pool without decreasing the development of all hematopoietic lineages.

**Adiposity induced by deficient melanocortin 4 receptor (MC4R) signaling accelerates thymic involution**

It is well known that deficient leptin signaling induces severe obesity and reduces thymic function ³⁴, ⁴⁰. However, leptin also exerts direct effects on thymopoiesis via functional leptin-receptor expression on thymus and T cells ³³, ³⁷-³⁹. Thus in leptin-deficient genetic model of obesity, the hyperphagia and metabolic disturbance induced by absence of leptin cannot be delineated from leptin’s direct prothymic and immunomodulatory effects. Therefore, in an effort to understand the effect of obesity induced via hyperphagia (independent of high-fat diet) through ablation of primary anorexigenic neural signaling, we investigated the Mc4r deficient mice maintained on ad libitum chow diet. This genetic model of obesity offers an additional advantage because Mc4r mRNA expression is restricted to CNS and hypothalamus and not expressed in thymic stromal cells, thymocytes, or mature T cells (Figure 5A). In addition, this model allowed us to determine whether adiposity independent of high-fat content of diet determines thymic function.

Mc4r mice (back crossed to more than 12 generation on C57BL/6 background)
and control wild type animals were maintained on chow diet and aged for 8 months. Mc4r<sup>−/−</sup> mice on ad libitum chow diet became progressively obese and weighed almost twice that of Mc4r<sup>+/+</sup> animals at 8 months of age (Supplementary Figure 1). Strikingly, the thymi of obese Mc4r<sup>−/−</sup> animals were grossly malformed and ‘fatty’ along with adipose tissue deposition in the mediastinal region (Figure 5B). The histological analyses of thymi revealed disruption of cortico-medullary junctions (CMJ) (Supplementary Figure 2).

We next investigated the impact of Mc4r driven obesity on thymopoiesis, splenic T cell subsets and TCR repertoire diversity. We found a marked reduction in naïve CD4<sup>+</sup> and CD8<sup>+</sup> cells in spleen of Mc4r<sup>−/−</sup> mice (Figure 5C, D). Also, Mc4r ablation resulted in selective expansion of CD8 E/M population with no change in CD4 E/M cells (Supplementary Figure 2). Consistent with reduced number of naïve T cells, the analysis of TREC in peripheral CD4 cells revealed a significant reduction in thymic output in Mc4r deficient animals (Figure 5E). Interestingly, the analysis of earliest thymocyte progenitors (ETP; Lin<sup>−</sup>CD117<sup>+</sup>CD44<sup>+</sup>) (Figure 5F) and LSK in BM (Figure 5G) revealed that obesity induced by Mc4r deficiency resulted in a significant decrease in lymphoid progenitors (Figure 5H).

We also determined the TCR repertoire by measuring the length of CDR3 hypervariable region using TCR spectratyping analysis. The PCR amplification of each Vβ allele by specific primer located in Cβ region and to every Vβ segment allows detection of CDR3 length of each Vβ- Jβ combinations<sup>47</sup>. We found marked perturbation in TCR repertoire of Mc4r<sup>−/−</sup> mice (Figure 6A). The significant disturbances were revealed in Vβ 15 and Vβ 18-21. Compared to lean WT mice which exhibited polyclonal repertoire, represented as Gaussian distribution of 6-8 peaks of each Vβ
family member, the obese Mc4r−/− animals exhibited more oligoclonal repertoire as evidenced by reduction in number and distribution of CDR3 lengths (Figure 6B). Consistent with our hypothesis, these findings demonstrate that hyperphagia and obesity driven by ablation of Mc4r recapitulates the reduced thymopoiesis and restriction of T cell diversity observed in DIO mice.

**Obesity reduces thymic output in middle-aged humans**

To understand the clinical implication of primary findings generated from mouse models, we next investigated the effect of adiposity on recent thymic emigrants (RTEs) in middle-aged (30-45 year old) humans. Quantitation of TRECs in peripheral blood mononuclear cells (PBMCs) revealed a strong negative correlation between the BMI and thymic output (Figure 7A). Since obesity is typically associated with insulin resistance, it prompted us to study whether that thymic involution observed in obese subjects is simply due to disturbance in glucose homeostasis or underlying type 2 diabetes (T2D). We therefore compared thymic output in lean healthy subjects (BMI = 20-24.9) with overweight (BMI = 25-30), overweight with T2D, obese (BMI = 31-40) with no T2D and obese with T2D as well as severely obese (BMI >45) subjects. The frozen PBMC from a total of 40 male and female subjects in each group with an age-range of 30-40 years were utilized for the TREC analysis. Interestingly, we found that healthy overweight or overweight subjects with T2D had similar thymic output as lean subjects (Figure 7B). However, compared to lean individual, obese subjects with or without T2D had significant reduction in TREC numbers and which were further diminished in severely obese individuals (Figure 7B). These set of data establish that progressive adiposity independent of T2D compromises thymopoiesis and development
of insulin-resistance in subjects with BMI >31 results in further reduction of recently generated thymic naïve T cells.
Discussion

Based on data from NHANES study, it is predicted that by year 2012, the prevalence of obesity is expected to reach 55% in African-American women, 36% in Caucasian females, 35% in Caucasian males and 33% in African-American men \(^{52,53}\). The latest data shows that by year 2010, there will 9.3 million more obese adults in US than last decade and 8.3 million of this newly emerging obese population will be constituted by adults that would be over the age of 50 years \(^{53}\). How “gerobesity” or prevalence of obesity in elderly will interact with ongoing corrosion of age-related immunity and mechanisms regulating immunosenescence mechanism remains to be determined. Currently, stress is thought to be one of the players in accelerating immunosenescence \(^{54}\). In this study we demonstrate that obesity compromises immune-surveillance pathways with accelerated age-related thymic involution, reduced naïve T cell production and restriction of TCR repertoire diversity.

Obesity is known to increase the risk of infections and certain cancers \(^{13,14}\). This epidemiological evidence gave rise to the hypothesis that chronic caloric excess may be responsible for an underlying immuno-deficient state with defects in mechanisms regulating the T cell generation in thymus \(^4\). We found that dietary-obesity selectively compromises the lymphoid progenitor pool while increasing the myeloid progenitors. Obesity induced by *ad libitum* intake of high fat diet in C57BL/6 male mice reduced thymopoiesis and restricted the TCR repertoire diversity. Furthermore, ablation of Mc4r mediated anorexigenic signaling and resultant obesity caused by excess caloric intake due to hyperphagia of chow diet recapitulates the defects in thymic function caused by high-fat diet induced obesity. Importantly, in humans, obesity reduced the thymopoiesis independently of frank type 2 diabetes (T2D). We demonstrate that
obesity induced acceleration of thymic aging is related with defects at multiple levels which include increased apoptosis of developing thymocytes, decrease in T cell precursor pool and reduction in recent thymic emigrants.

The ability to generate effective T cell response to newly emerging pathogens is dependent on a broad T cell repertoire 8 of newly generated thymic emigrants. The restriction of TCR repertoire is typically seen in elderly because of inability of thymus to replenish the naïve T cell pool 9. Consequently, the risk of infections, cancer and vaccination failures increase upon aging 10, 11. According to current estimates, approximately $3 \times 10^9$ T cells have to be generated everyday to replenish the total pool of existing $3 \times 10^{11}$ T cells in human body 49. Because by 40 years of age, approximately 80% of thymic space is dysfunctional and composed of adipose tissue 55, maintenance of TCR repertoire and peripheral naïve T cell pool is largely thymic-independent 49. The memory T cells have greater homeostatic proliferation rate than naïve cells and because of ongoing antigen exposures, the integrity of naïve T cell niche is progressively eroded which leads to restricted TCR repertoire and reduced immune-surveillance 8, 48, 49. Recent studies in diet-induced obese (DIO) mice, a relevant disease model to mimic human dietary-obesity, provide evidence that DIO animals are prone to mortality upon influenza infection and exhibit defective immune response 16, 19-23. Furthermore, obese individuals have increased risk of mortality and morbidity as a result of severe infections and are also prone to nosocomial infections 14, 15. Therefore, restriction of TCR repertoire diversity as a result of obesity induced thymic aging provides a mechanistic link to greater risk of infections and lower immune-surveillance in obese individuals. Consistent with possible defects in T cell compartment, it has been suggested that obese individuals are lymphopenic, and their T cells exhibit reduced
proliferation in response to mitogens which can be corrected by weight loss 56.

Age-related thymic involution is known to be associated with significant decrease in TECs, increase in adipogenic-fibroblasts and deterioration of thymic stromal microenvironment 3, 9, 26, 55. Our recent studies suggest that caloric restriction, anti-thesis of dietary obesity, prevents age-related loss of TECs and increases in fibroblasts with reduction in epithelial-mesenchymal transition (type 2 EMT) regulators in thymus 3, 57. Therefore, further studies are necessary to determine whether obesity alters TECs or stromal cell microenvironment that regulates T cell development and thymopoiesis. How diet-induced obesity accelerates thymic involution process is incompletely understood, and considering the broad effects of obesity, it is likely that mechanisms involved are complex and multifactorial. Increased caloric intake due to hyperphagia is known to lead to obesity. Accordingly, deficient anorexigenic neural drive seen in leptin (ob/ob) and leptin-receptor mutant (db/db) mice as well as loss of melanocortin 4 receptor signalling are known to cause obesity 40. We have previously reported that leptin-receptors are expressed on T cells and leptin can directly promote thymic function in aging mice 33, 37, 38. Thus, the absence of leptin signaling may induce thymic involution independently of adiposity and hyperphagia. Interestingly, the ablation of Mc4r also increases food intake with rapid onset of obesity 42-44. However, unlike leptin-receptors, Mc4r is not expressed in bone marrow, thymus, TSCs or T cells and its expression is restricted to CNS. Therefore the observed effects on thymic function in obese Mc4r null mice are likely mediated via the loss of CNS anorexigenic drive and resultant adiposity. Consistent with our hypothesis, the data suggest that similar to DIO mice the reduction of thymopoiesis in obese Mc4r animals is linked with diminished lymphoid progenitor pool. Furthermore, loss of Mc4r signaling mediated adiposity and
acceleration of thymic involution led to perturbation and premature restriction of TCR repertoire diversity by middle age in mice. The Mc4r\textsuperscript{+/−} did not display any thymic defects (data not shown) suggesting that homozygous and complete loss of function mutation and development of obesity is required for thymic involution. Given the loss of function mutation in Mc4r is the most prevalent monogenic cause of human obesity\textsuperscript{40}; our findings from Mc4r null mouse models may warrant a careful evaluation in humans to determine whether homozygosity of Mc4r mutation accelerates thymic aging and immunosenesence.

Our findings describe previously unrecognized relationship between obesity, thymic aging and immunosenesence with implications for mechanisms governing health-span. Consistent with our data from animal models, thymopoiesis in humans is inversely related to BMI. Therefore, reduced thymic function in middle-aged obese individuals with and without type 2 diabetes can potentially be an important mechanism of reduced immune-surveillance. According to current predictions, by year 2030 approximately 1 in 8 people will be over the age of 65\textsuperscript{9}. Evidence suggests that within the next 5 years, 1 in 4 people over the age of 50 in US will be obese\textsuperscript{53}. It is well known that age-dependent reactivation of herpes zoster infections, increased morbidity and mortality from influenza and other infections along with partial protection by vaccines significantly limit the health-span\textsuperscript{8,9}. With the changing demographics of the aging US population and increasing obesity rates in elderly, the reduced immune competence in this group is an emerging health concern. Presently, most of ongoing research efforts to ameliorate immunosenesence are directed towards rejuvenating the naïve T cell production from thymus without significant consideration to adiposity and with the assumption that elderly population will either be of normal BMI or frail upon advanced
aging. Our data suggest that obesity induced accelerated thymic involution and restriction of T cell repertoire diversity represents a potent modifier of immunosenescence mechanisms which may further increase the risk and severity of infections in “gerobese” population with potentially greater predisposition to emerging diseases like H1NI influenza, West Nile Virus or Severe Acute Respiratory Syndrome (SARS). Understanding the basic mechanism of adipose-immune interactions of obesity and immunosenescence may lead to development of approaches for successful immunological aging and increased health-span. Our data establish that obesity accelerates the aging of T cell compartment by inducing defects in thymic function.
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Authorship

HY and YHY conducted the experiments, analyzed the data, and participated in study design and manuscript preparation. BV and KGK, conducted the experiments and analyzed the data. JR participated in study design and data analysis. AAB contributed the knockout mouse model and participated in study design and data analysis. VDD, planned the study, conducted experiments and wrote the paper.

Conflict of Interest Disclosure

All authors have no conflicting financial interests.
References


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Figure Legends

**Figure 1: Dietary-obesity induces thymic involution.** (A) Compared to chow fed control male mice, the thymus appeared ‘fatty’ in age matched 13 mo old DIO animals along with increased body weight and reduced total thymic cell numbers (n = 12 per group). (B) The hematoxylin and eosin stained sections of 13m old control and DIO mice revealed increased perithymic fat and obliteration of CMJ (C denotes cortex, M is Medulla). (C) FACS analysis of CD4-FITC and CD8-PE stained thymocytes. The 13m old DIO mice had significant reduction in single positive CD4, CD8 and double positive cells. (D) The annexin-V staining on CD4 SP and CDSP cells revealed statistically significant (p <0.05) increase in frequency of apoptosis. Data is expressed as mean (SEM) from 8-12 mice per group.

**Figure 2: Obesity decreases naïve T cells and expand memory cells.** Chow fed control and high fat (60%) fed mice were evaluated for naïve (CD62L⁺CD44⁻, red box) E/M (CD62L⁻CD44⁺) CD4 and CD8 T cells at 3 months (n = 10-12/group), 9month (n = 8/group) and 13month of age (n = 12/group). Obesity significantly reduces naïve cells and expands effector/memory splenic T cells in 9 and 13m old mice but not at 3m of age.

**Figure 3: Dietary obesity reduces thymopoiesis and restricts TCR diversity.** (A) The splenic CD4⁺ T cells were isolated to prepare the DNA and signal-joint TREC levels were analyzed using quantitiative PCR analysis. A total of 6-10 mice per group were utilized for sjTREC assay and the data is expressed as mean ± SEM. The obesity in mice significantly reduced TREC at all ages examined. (B) Real-time PCR analysis
of cytokine mRNA expression in purified CD4 cells from 13m old control and DIO mice. (n = 6/group).(C) The TCR spectratyping analysis of CD4 T cells from 3 and 9m old mice on chow and high-fat diet is shown (n = 5). A polyclonal profile is Gaussian with 6-8 peaks while alterations from Gaussian distributions are measure of oligoclonality. The Gaussian distribution profiles were translated into probability distributions as functions of the area under the curve for each CDR3 length. The average distribution of the CD4+ repertoire from 3 and 9 month old ad libitum fed chow diet controls is compared to the DIO mice. The statistical quantitation of the CDR3 size of all the TCR Vβ between control and DIO mice was performed using CDR3QAssay software. The extent of the change in the CDR3 size distribution is defined as percent improvement (distance from the mean value. The % Improvement > 3 standard deviations in the fragment length of each family indicates that there are significant changes in the Vβ family ) (based on Gorochov et al ref. 47). These improvements in TCR diversity are represented as landscape surfaces, in which smooth (blue) landscapes represent an unchanged TCR repertoire (diversity). The Mountain (in green, yellow and orange) depicts perturbation in amplified peaks of CDR3 lengths compared to control mice. Each line crossing on the y axis of the landscape denotes perturbation for a specific CDR3 length or size (x-axis) of a particular Vβ family (z-axis).

**Figure 4: Obesity reduces lymphoid pool and increases myeloid progenitors.** (A) Bone marrow cells from femur of control and DIO mice (13m, n = 6/group) were stained for lineage markers, CD117, Sca1 and CD127. A representative dot plot show gating strategy (on Linlo cells) and Flt3 expression to define MPP (Flt3+LSK) and CLPs
(Lin−Sca1−CD117hiCD127+ with CD127 expression depicted as histogram. (B) Flow cytometry analyses of BM cells from 13m old control and DIO mice. Obesity increases the subsets of common myeloid progenitor (Lin−CD127−CD117hiSca1 CD34+CD16CD32−), megakaryocyte-erythrocyte progenitor (Lin−CD127−CD117hiSca1− CD34−CD16CD32−) and significantly reduced (P <0.05) granulocyte-monocyte progenitor (Lin−CD127−CD117hiSca1−CD34+CD16CD32+).

**Figure 5: Deficient Mc4r signaling driven obesity accelerates thymic involution.**

The WT and Mc4r knock out mice were maintained on *ad libitum* chow diet and aged for 8 months. (A) The real-time PCR analysis of Mc4r mRNA in 2-3 month old C57BL/6 mice (n =3). The total RNA from cells and tissues was DNAse digested and RT-PCR analysis shows that CNS restricted expression of Mc4r. (B) Obesity mediated via in Mc4r deficiency causes thymic adiposity. Thymus is highlighted in blue box and arrows, compared to control WT mice, inset shows malformed fatty thymus in Mc4r−/− animals. (C-D) Compared to WT mice, loss of Mc4r significantly (p<0.05) reduces the frequency of CD4 and CD8 naïve (CD62L−CD44−) T cells and (E) decreases sjTREC numbers in splenic CD4 cells. (F, H) Mc4r deficiency driven obesity significantly reduces the ETP (Lin−loCD44+ckithi ) cells in thymus and (G, H) LSK(Lin−Sca1+Kit+) in bone marrow (n = 5 per group).

**Figure 6: Mc4r mediated adiposity induces premature restriction of TCR diversity.**

(A) The TCR spectratyping analysis of CD4 T cells from control and Mc4r null mice is shown. The x-axis shows CDR3 lengths, and is each line crossing on the y axis of the landscape denotes perturbation for a specific CDR3 size (x- axis) while individual Vβ
are shown on (z-axis). The mountain (in green, yellow and orange) depicts perturbation in amplified peaks of CDR3 lengths of MC4R\textsuperscript{-/-} animals in comparison to WT mice (n = 5). (B) Representative Vβ results of CDR3 size spectratyping in WT and Mc4r null mice.

**Figure 7: Obesity in humans reduces thymopoiesis** (A) Obesity in humans reduces thymopoiesis, quantitative PCR analysis of human sjTREC in PBMCs shows significant correlation between reduction in TREC with increasing BMI (30-40 years old) (B) The frozen PBMCs from additional, (male and female n = 35-30; age 30-45 years), lean subjects were analyzed for TREC and compared with overweight and obese subjects with and without type 2 diabetes. All morbid obese (BMI >45) were insulin-resistant.
Figure 1, Yang et al
Figure 2, Yang et al
Figure 3, Yang et al
Figure 4, Yang et al
Figure 5, Yang et al.
Figure 6, Yang et al
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Obesity accelerates thymic aging

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