# Obesity-Induced Changes in T-Cell Metabolism Are Associated With Impaired Memory T-Cell Response to Influenza and Are Not Reversed With Weight Loss

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***Background.*** Obesity is an independent risk factor for increased influenza mortality and is associated with impaired memory T-cell response, resulting in increased risk of infection. In this study, we investigated if weight loss would restore memory T-cell response to influenza.

***Methods.*** Male C57BL/6J mice were fed either low-fat or high-fat diet to induce obesity. Once obesity was established, all mice received primary infection with influenza X-31. Following a recovery period, we switched half of the obese group to a low-fat diet to induce weight loss. Fifteen weeks after diet switch, all mice were given a secondary infection with influenza PR8, and memory T-cell function and T-cell metabolism were measured.

***Results.*** Following secondary influenza infection, memory T-cell subsets in the lungs of obese mice were decreased compared to lean mice. At the same time, T cells from obese mice were found to have altered cellular metabolism, largely characterized by an increase in oxygen consumption. Neither impaired memory T-cell response nor altered T-cell metabolism was reversed with weight loss.

***Conclusion.*** Obesity-associated changes in T-cell metabolism are associated with impaired T-cell response to influenza, and are not reversed with weight loss.

**Keywords.** influenza; obesity; T cells; memory; metabolism; weight loss.

The 2009 H1N1 influenza pandemic introduced obese adults as a vulnerable population to influenza virus infection. For the first time, obesity was recognized as an independent risk factor for increased morbidity and mortality [1]. This is concerning, as over 37% of the adult US population is obese [2] and seasonal influenza infection remains in the top 10 causes of death in the United States [3, 4].

Models using diet-induced obese mice and human subjects demonstrate impairments in both innate and adaptive immune responses following influenza infection or vaccination [5–14]. Diet-induced obese mice have increased mortality to primary [13] and secondary [5] influenza infections, impaired maintenance of influenza-specific memory T cells [6], and decreased memory T-cell function [5]. In a human influenza vaccine study, peripheral blood mononuclear cells (PBMCs) from both overweight and obese adults have decreased $CD4^+$ and $CD8^+$ T-cell activation and function [9, 10]. Alarmingly, despite influenza vaccination, obese adults have twice the risk of developing influenza or influenza-like illness compared with vaccinated healthy-weight adults [15]. However, the mechanism by which obesity impairs the T-cell response to influenza remains unknown.

T-cell function and metabolism are closely linked and changes in T-cell metabolism can alter function. In general, naive T cells utilize oxidative metabolism to fuel immune surveillance whereas activated effector T cells switch to a metabolic phenotype characterized by aerobic glycolysis and glutamine oxidation essential for effector function, growth, and proliferation [16]. The conversion of effector T cells to long-lived memory T cells requires a switch back to oxidative metabolism, with fatty acids as the predominate fuel source [17, 18]. Given obesity results in systemic metabolic dysregulation of glucose usage and fatty acid oxidation and storage [19, 20], we hypothesized obesity would impair T-cell metabolism, leading to memory T-cell dysfunction in response to influenza infection. Furthermore, we hypothesized restored systemic metabolism via weight loss would be sufficient to recondition T-cell metabolism and thereby improve T-cell memory responses to infection.

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We used a mouse model of influenza infection, where lean and diet-induced obese mice were inoculated with X-31 influenza virus to generate memory T cells, followed by weight loss induced by a diet switch. After significant weight loss and normalization of serum glucose and insulin, mice were reinfected with PR8 influenza virus, and T-cell metabolism and function were assessed. For the first time, our results demonstrate obese mice have impaired T-cell metabolism, concomitant with impaired memory T-cell responses to influenza infection. Furthermore, we show that despite significant weight loss, formerly obese mice retain impaired T-cell metabolism and impairments in memory T-cell function upon secondary reinfection. These results suggest that obesity-associated changes in T-cell metabolism may be responsible for impaired T-cell memory response to influenza.

## MATERIALS AND METHODS

### Mice and Diets

C57BL/6J 6-week-old male mice were obtained from The Jackson Laboratory and allowed 1 week of acclimation. Mice were group housed (3–5 per cage), maintained at ambient temperature, and given ad libitum access to food and water. Thirty mice were placed on low-fat chow diet (LFD; Harlan Laboratories, 2920X) with 60 mice placed on 60% high-fat diet (HFD; Research Diets, D12492) for 22 weeks. Diet-induced obese mice were then split into 2 groups, with 30 mice remaining on HFD and 30 mice switched to LFD to induce weight loss. Mice were maintained on the weight-switched diets for 15 weeks. All procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill.

### Influenza Infections

For primary influenza infection, mice were lightly anesthetized with isoflurane and infected intranasally with 30 µL of sterile phosphate-buffered saline (PBS) containing 400 egg 50% infectious dose of live X-31 mouse-adapted recombinant influenza virus strain that consists of external hemagglutinin and neuraminidase proteins of A/Aichi/2/68 (H3N2) [21] and

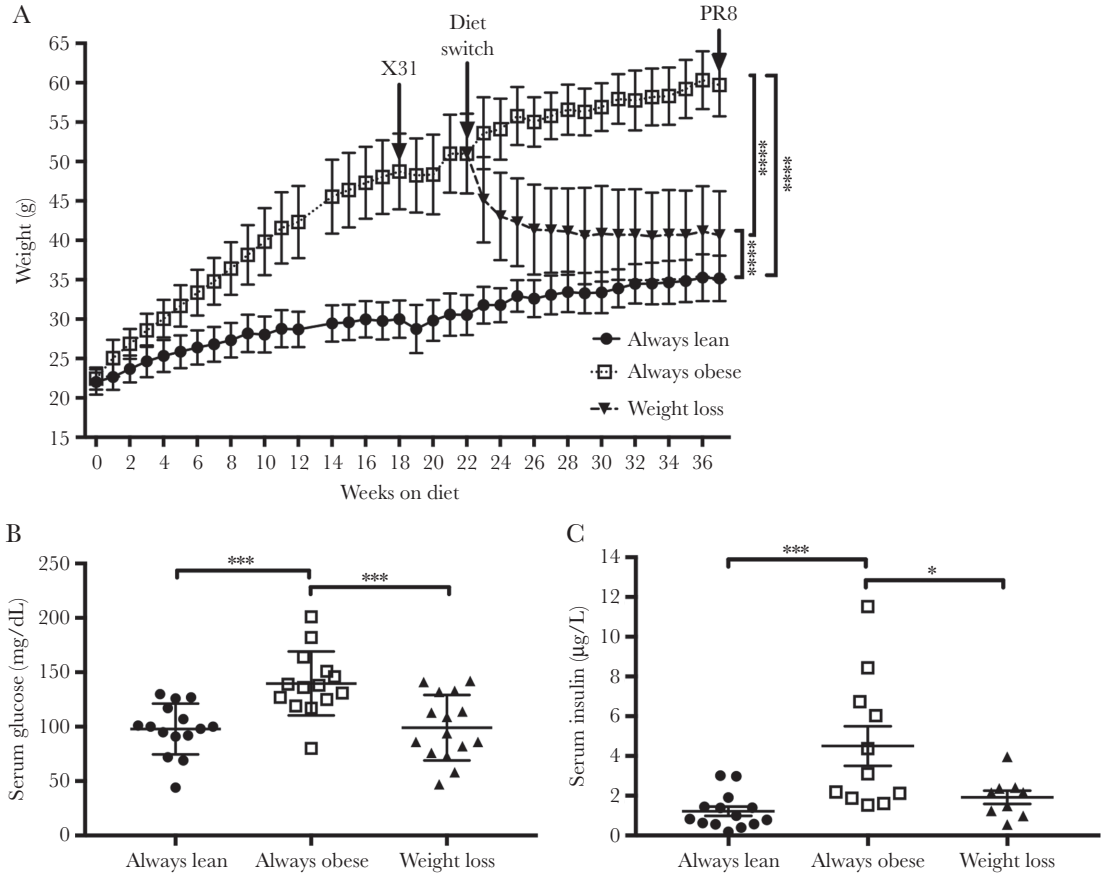


**Figure 1.** Weight loss restores serum glucose and insulin levels in formerly obese mice. *A,* Male, 7-week-old C57BL/6J mice were fed low-fat (n = 30) or high-fat diet (n = 60) for 18 weeks. Mice were infected with X-31 influenza virus for generation of memory T cells (4 weeks). Four weeks following primary infection, diets were switched and half of the mice receiving high-fat diet (n = 30) were placed on low-fat diet (n = 30). Mice were maintained on switched diet for 15 weeks and then infected with PR8 influenza virus. Body weights were measured weekly. *B,* Fasting serum glucose and (*C*) serum insulin of always lean, always obese, and weight loss mice prior to secondary PR8 infection. Each symbol represents data obtained from 1 mouse with mean ± standard deviation (*A, B*), or mean ± standard error of the mean (*C*). Two-way ANOVA (*A*) and 1-way ANOVA (*B, C*) with Tukey multiple comparisons were used to compare groups. **P* < .05, ****P* < .001, *****P* < .0001.

the internal proteins of A/Puerto Rico/8/34 (PR8). Mice were weighed daily for 14 days to monitor weight loss. For secondary infection, mice were infected intranasally with 30 µL of sterile PBS containing 0.5 hemagglutination units of PR8 (American Type Culture Collection, Manassas, VA), a H1N1 influenza virus. Mice were then sacrificed at day 0, 3, and 7 after secondary infection with PR8.

#### Lung Viral Titers

Lung viral titers were determined using a modified 50% cell culture infectious dose protocol using hemagglutination as an endpoint, as previously described [8].

#### *Bronchoalveolar Lavage Total Protein*

Lungs were lavaged with Hanks' Balanced Salt Solution, and the collected supernate used for measurement of total protein, as previously described [8].

#### *Lung Histopathology*

Lungs were removed and inflated with 4% paraformaldehyde fixative, paraffin embedded, and stained with H&E. Determination of lung pathology was performed as previously described [8].

#### Fasting Glucose and Insulin

One week prior to secondary infection (week 37 on diet), mice were fasted for 6 hours, blood was collected by tail nick, and glucose levels were measured by glucometer (Freestyle). At time of sacrifice, serum was collected and insulin levels were measured by enzyme-linked immunosorbent assay (Mercodia).

#### Antibodies and Flow Cytometry

Preparation of lungs and visceral adipose tissues for flow cytometry analysis and staining protocols have been described elsewhere [8, 22]. The following antibodies and reagents were used:

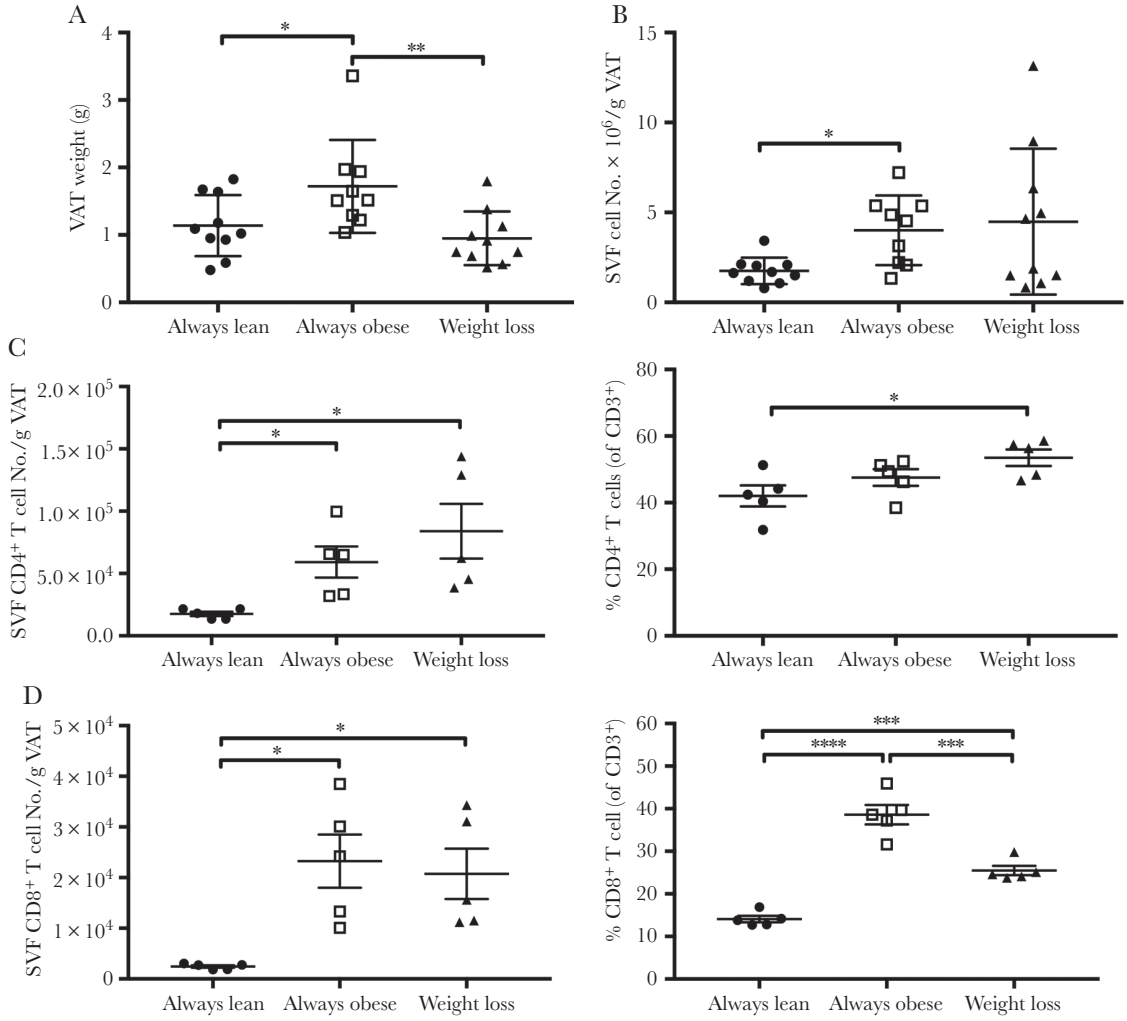


**Figure 2.** Weight loss does not reverse visceral adipose tissue (VAT) T-cell infiltration in formerly obese mice. *A*, Epididymal visceral adipose tissue (VAT) weights and (*B*) stromal vascular fraction (SVF) cell number per gram VAT was determined prior to secondary PR8 infection. *C*, $CD4^+$ T-cell number and (*D*) $CD8^+$ T-cell number were determined from total SVF cells collected prior to secondary PR8 infection. Each symbol represents data obtained from 1 mouse with mean ± standard deviation (*A*, *B*) or mean ± standard error of the mean (*C*, *D*). One-way ANOVA with Tukey multiple comparisons were used to compare groups. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$.

Alexa Fluor 700 anti-mouse CD3 (17A2, eBioscience), APC-Cy7 rat anti-mouse CD4 (GK1.5, BD Pharmingen), PacBlue rat anti-mouse CD8a (53–6.7, BD Pharmingen), PerCP-Cy5.5 rat anti-mouse CD11a (2D7, BD Pharmingen), FITC rat anti-mouse CD25 (3C7, BD Pharmingen), APC anti-mouse Foxp3 (FJK-16s, eBioscience), BUV395 hamster anti-mouse CD69 (H1.2F3, BD Biosciences), BV480 rat anti-mouse CD103 (M290, BD Biosciences), Zombie Yellow Fixable Viability Dye (BioLegend), Alexa Fluor anti-mouse 594 CD4 (GK1.5, Biolegend), BUV395 rat anti-mouse CD8 (53–6.7, BD Biosciences), FITC rat anti-mouse CD62L (MEL-14, BD Biosciences), APC anti-mouse Granzyme B (GzA-3G8.5, eBioscience), PE-Cy7 anti-mouse interferon gamma (XMG 1.2, eBioscience), Zombie NIR (Biolegend), BUV395 rat anti-mouse CD4 (GK1.5, BD Biosciences), BV650 rat anti-mouse CD8a (53–6.7, BD Biosciences), PerCP-Cy5.5 rat anti-mouse CD62L (MEL-14, BD Biosciences), PE-Cy7 anti-mouse CCR7 (4B12, eBioscience), BV421 rat anti-mouse CD127 (SB/199, BD Biosciences), BB515 rat anti-mouse CD44 (IM7, BD Biosciences), and class II tetramer I-A(b) Influenza A NP 311–325 (QVYSLIRPNENPAHK, courtesy of National Institutes of Health Tetramer Core Facility). All samples were acquired on a BD LSR II flow cytometer, and data were analyzed using FlowJo (Treestar).

#### Extracellular Acidification Rate and Oxygen Consumption Rate

T cells were isolated from mouse splenocytes on day 0 and day 7 after PR8 infection. Cells were isolated using magnetic bead negative selection (Miltenyi) for $CD4^+$ and $CD8^+$ T cells in MACS buffer (PBS + 0.5% fetal bovine serum + 2 mM ethylenediaminetetraacetic acid). Isolated T cells were counted using Bio-Rad TC20 with trypan blue exclusion for viability. XFe96 cell culture microplates were treated with Cell-Tak (Corning) in 0.1 M sodium bicarbonate to allow for cell adherence. $CD4^+$ and $CD8^+$ T cells were plated in nonbuffered RPMI-1640 with freshly added 10 mM glucose and 2 mM glutamine at 150 000 cells per well. Extracellular acidification (ECAR) and oxygen consumption rates (OCR) were determined using the Seahorse XFe96 Flux analyzer (Agilent) at 37°C, as previously described [23]. OCR and ECAR were normalized to cell number.

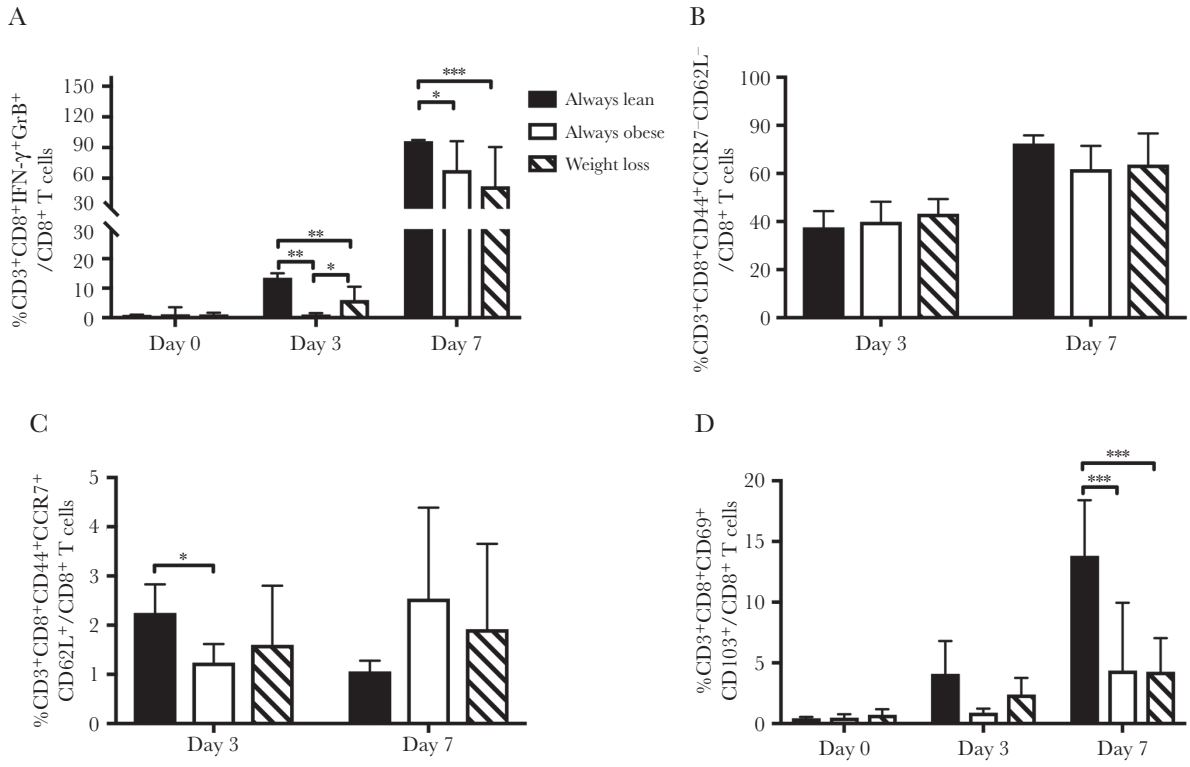


**Figure 3.** Obesity, regardless of weight loss, results in dysregulated generation and function of $CD8^+$ memory T-cell populations to influenza infection. Lungs were harvested, digested, and homogenized into single-cell suspensions and $1 \times 10^6$ cells were stained for $CD8^+$ effector and memory T-cell populations and function by flow cytometry. *A*, Percent $CD8^+$ effector T cells expressing intracellular functional markers granzyme B (GrB) and interferon-gamma (IFN-γ) at days 0, 3, and 7 after PR8 infection in always lean, always obese, and weight loss groups (n = 4–5). *B*, Percent of $CD8^+$ effector memory T-cell populations at days 0, 3, and day 7 after PR8 infection (n = 4–5). *C*, Percent of $CD8^+$ central memory T cells comparing day 3 and 7 after PR8 infection (n = 4–5). *D*, Percent of $CD8^+$ tissue-resident memory T-cell populations comparing days 0, 3, and 7 after PR8 infection (n = 4–5). Each bar represents the mean ± standard error of the mean. One-way ANOVA with Tukey multiple comparisons was used to compare groups at each time point. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$.

### Statistical Analysis

Details describing the statistical analysis and sample size of each experiment can be found in the figure legends. Potential outliers were assessed using ROUT with Q equal to 1%. All statistical analysis was performed using GraphPad Prism 7 for Mac OS X, version 7.0c (GraphPad Software, Inc., La Jolla, CA). All data were determined as significant by $P < .05$.

## RESULTS

### Model to Study the Effects of Weight Loss on Memory T Cells

We utilized a well-established mouse model for both influenza infection and obesity [24–26]. Male 7-week-old C57BL/6J mice were placed on either a LFD (n = 30) or a 60% HFD (n = 60) for 18 weeks. As expected, mice fed 60% HFD gained significantly more weight than LFD fed mice (Figure 1A).

Following 18 weeks on their respective diets, mice were infected with influenza X-31 and maintained their diet for an additional 4 weeks, allowing T-cell memory to develop in either the lean or obese state. After memory generation, half of the obese mice were switched to LFD, leaving 30 obese mice remaining on HFD. This created 3 groups of mice, which we termed: (1) always lean, (2) always obese, and (3) weight loss. Mice were maintained on the indicated diets for an additional 15 weeks. As shown in Figure 1A, obese mice switched from HFD to LFD (weight loss group) had a significant difference in final body weight compared to the always obese group.

Always obese mice developed hyperglycemia (Figure 1B) and hyperinsulinemia (Figure 1C), indicating systemic insulin resistance as a consequence of obesity. Both always lean and weight loss mice had significantly lower fasting serum glucose (Figure 1B) and serum insulin levels (Figure 1C) compared to always obese mice, with no difference between always lean and weight loss groups. Thus, mice that were previously obese but then lost weight developed a similar systemic metabolic phenotype to the always lean mice.

As expected, always obese mice had greater visceral epididymal fat pad mass, which was significantly reduced with weight loss, indistinguishable from that of always lean mice (Figure 2A). Additionally, always obese mice had higher numbers of infiltrating cells in the stromal vascular fraction (SVF) of the visceral fat pad compared to always lean mice. Interestingly, weight loss did not reduce stromal vascular cell numbers, as there was no difference between always obese and weight loss groups (Figure 2B). Using flow cytometry, we identified T-cell populations within the SVF. $CD4^+$ and $CD8^+$ T cells were

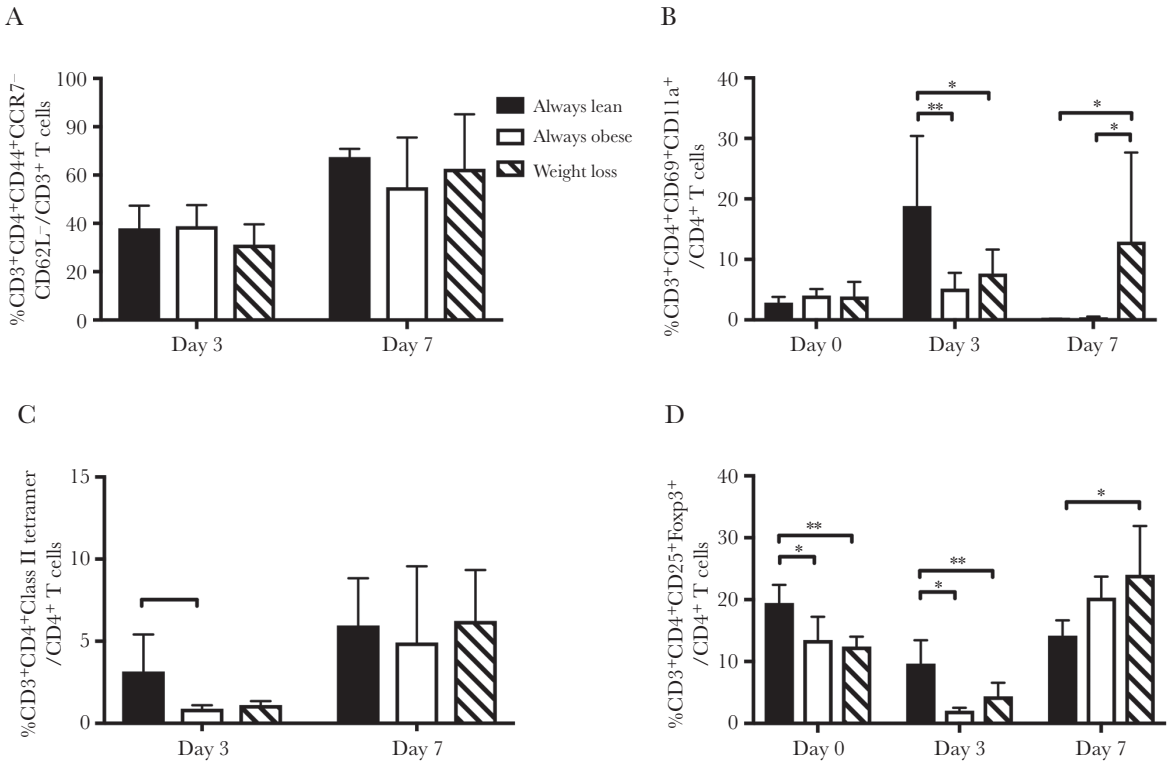


**Figure 4.** Obesity, regardless of weight loss, results in dysregulated generation and function of $CD4^+$ memory T-cell populations to influenza infection. Lungs were harvested, digested, and homogenized into single-cell suspensions and $1 \times 10^6$ cells were stained for $CD4^+$ memory, influenza-specific, and regulatory T-cell populations and function by flow cytometry. *A*, Percent of $CD4^+$ effector memory T-cell populations at day 3 and day 7 postinfection to PR8 (n = 4–5). *B*, Percent of $CD4^+$ tissue-resident memory T cells at days 0, 3, and 7 after PR8 infection (n = 4–5). *C*, Percent of class II tetramer I-A(b) influenza A NP 311–325 positive cells at days 3 and 7 after PR8 infection (n = 4–5). *D*, Percent of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells at days 0, 3, and 7 after PR8 infection (n = 4–5). Each bar represents the mean ± standard error of the mean. One-way ANOVA with Tukey multiple comparisons was used to compare groups at each time point. $^*P < .05$, $^{**}P < .01$.

greater in both always obese and weight loss groups compared with always lean mice (Figures 2C and 2D). Differences in $CD4^+$ T cells expressing interferon-gamma (IFN-γ) and interleukin-17 (Th1 and Th17 cells, respectively) and in $CD8^+$ T cells expressing IFN-γ were also found in visceral adipose tissue SVF among always lean, always obese, and weight loss groups (Supplementary Figure 1A–1C).

Lung pathology, viral titers, and lavage fluid total protein (Supplementary Figure 2A–2C) were equivalent among all 3 groups of mice at day 3 and day 7 postinfection. We have previously reported differences in lung pathology in lean versus obese mice only when using a higher infectious dose of PR8 that resulted in significant mortality in obese mice [5, 8, 14].

#### Weight Loss Does Not Restore Memory T-Cell Populations

At 15 weeks after diet switch, all mice were reinfected with H1N1 PR8. Primary infection with X-31, followed by secondary infection with PR8, provides a model for testing T-cell memory responses specifically, as the external hemagglutinin (HA) and neuraminidase (NA) proteins of X-31, an H3N2 virus, generate antibody that cannot neutralize the HA and NA proteins of PR8, an H1N1 virus. However, because internal viral proteins are the same between the 2 viruses, infection with X-31 will generate a robust memory T-cell response that can protect from a lethal infection with PR8.

Resolution of primary influenza infection results in approximately 90%–95% of effector T-cell death, with the remaining T cells acquiring a long-lived memory phenotype [21]. We identified $CD8^+$ T-cell subsets in the lungs of mice at 0, 3, and 7 days after PR8 infection in always lean, always obese, and weight loss mice (Figure 3A–3D). As shown in our previous studies [5, 6], obese mice had a lower percent (Figure 3A) and number (Supplementary Figure 3A) of $CD8^+$ T cells expressing IFN-γ and Granzyme B (GrB) at both days 3 and 7 after PR8 infection. Although mice in the weight loss group were challenged with PR8 in a lean state, their memory T-cell populations were generated with X-31 while in an obese state. Interestingly, weight loss did not restore functional $CD8^+$ effector T-cell populations, with significantly lower percent of $CD8^+IFN\text{-}\gamma^+GrB^+$ cells at both days 3 and 7 (Figure 3A).

Next, we identified effector and central memory $CD8^+$ T cells: Tem (Figure 3B) and Tcm (Figure 3C), respectively. There were no differences in percent of Tem between groups at either day 3 or 7, yet there were significantly lowers numbers of Tem cells in both always obese and weight loss mice at day 7 (Supplementary Figure 3B). Always obese mice had decreased percentage of $CD8^+$ Tcm cells at day 3 postinfection (Figure 3C), and decreased cell numbers at day 7 (Supplementary Figure 3C). Importantly, we also examined $CD8^+$ resident memory T cells (Trm) in the lung, which have recently emerged as a critical population of T cells for protection against influenza reinfection. At day 7 postinfection, compared to always lean mice, always obese and weight loss mice had significantly lower percent $CD8^+$ Trm (Figure 3D) and cell number (Supplementary Figure 3D). Thus, weight loss had no effect in restoring this critical population.

In addition to examining $CD8^+$ T-cell populations, we also identified $CD4^+$ Tem, Trm, and T regulatory cells (Treg). Although we found no significant differences in the percent of $CD4^+$ Tem among groups at any time point (Figure 4A), there were lower absolute numbers in always obese mice at day 7 (Supplementary Figure 4A). Percent $CD4^+$ Trm were significantly lower day 3 postinfection in always obese mice, and were not restored with weight loss (Figure 4B). Interestingly, the

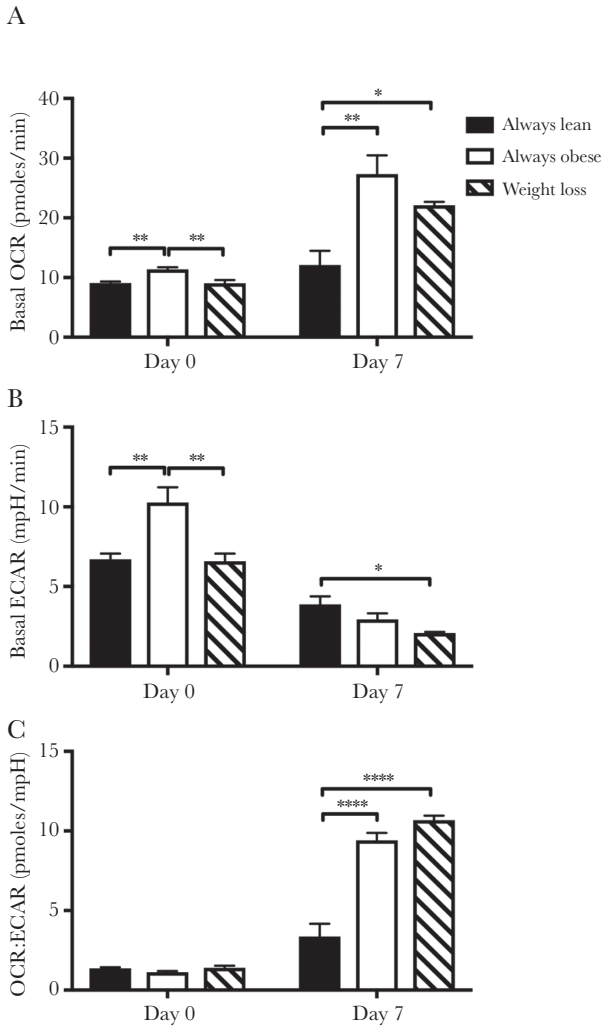


**Figure 5.** Altered $CD4^+$ T-cell metabolism is not reversed by weight loss. Spleens harvested from mice were used for $CD4^+$ T-cell isolation by negative selection magnetic bead separation. Cells were plated at 150 000 cells per well, and extracellular flux analysis was performed using the Seahorse XFe96 Flux analyzer at 37°C. *A*, Basal oxygen consumption rate (OCR) as a surrogate for mitochondrial respiration, (*B*) basal extracellular acidification rate (ECAR) as a surrogate for glycolysis, and (*C*) the ratio of oxidation to glycolysis (OCR:ECAR) were determined in $CD4^+$ T cells at day 0 and day 7 after PR8 infection in always lean, always obese, and weight loss mice (n = 5 per group). Data represent mean ± standard error of the mean. One-way ANOVA with Tukey multiple comparisons were used at day 0 and day 7 to compare differences between groups. $^*P < .05$, $^{**}P < .01$, $^{****}P < .0001$.

weight loss mice had a significant increase in the percent and absolute numbers (Supplementary Figure 4B) of this population at day 7.

To measure influenza-specific $CD4^+$ T cells, we utilized a class II influenza tetramer specific for influenza A $NP_{311-325}$. We found that at day 3 postinfection, always obese mice had significantly fewer percentage of influenza-specific $CD4^+$ T cells in their lungs (Figure 4C), with weight loss mice nonsignificantly lower ($P$ = .0643). Total cell numbers did not differ (Supplementary Figure 4C), nor were there differences in influenza-specific $CD4^+$ T cells among groups at day 7.

Finally, Treg cells are a critical population involved in reducing the inflammatory response after influenza clearance [27]. We found the percent of $CD4^+$ Treg cells were significantly lower in always obese mice at days 0 and 3 postinfection, and were not restored with weight loss (Figure 4D). At day 7 postinfection, compared with always lean, weight loss mice had an increased percent of Treg cells, although absolute numbers did not differ among groups (Supplementary Figure 4D).

These results indicate that memory T-cell subsets are dysregulated when generated in an obese state, and that weight loss following generation of these populations does not restore memory T-cell populations to the lean state.

#### $CD4^+$ and $CD8^+$ T-Cell Metabolism Is Impaired in the Obese State and Is Not Restored by Weight Loss

Oxidative and glycolytic metabolism were determined using an Agilent Seahorse extracellular flux analyzer, which measures the change in ECAR (as a surrogate for lactate production and glycolysis) and the change in OCR (as a surrogate for mitochondrial respiration). ECAR and OCR were measured in splenic $CD4^+$ and $CD8^+$ T cells isolated at day 0 (uninfected) and day 7 after PR8 reinfection. Compared to always lean mice, $CD4^+$ T cells from always obese mice had significantly greater OCR at day 0 (prior to secondary infection; Figure 5A), and dramatically greater OCR at day 7 postinfection (Figure 5A). Weight loss mice had equivalent $CD4^+$ T-cell OCR to always lean mice prior to reinfection (day 0). However, at day 7 postinfection, OCR was significantly greater in weight loss mice compared with always lean, and did not differ from always obese (Figure 5A). Although there was no significant increase in OCR in $CD4^+$ T cells from day 0 to day 7 in always lean mice, there was a substantial increase in OCR in always obese and weight loss groups in response to secondary infection (Table 1).

$CD4^+$ T cells from always obese mice had significantly greater ECAR at day 0 compared to always lean and weight loss groups (Figure 5B). However, at day 7 postinfection, ECAR was significantly lower in the weight loss group compared with always lean. All 3 groups of mice had a significant drop in ECAR at day 7 postinfection (Table 1).

Ratios of basal OCR:ECAR, a measure of relative magnitude of mitochondrial oxidation versus glycolysis, were determined at day 0 and day 7 postsecondary infection. As seen in Figure 5C, there were no OCR:ECAR differences prior to secondary infection. However, following infection, always obese and weight loss mice had significantly greater OCR to ECAR compared to always lean mice, demonstrating an overall change in T-cell metabolism in obesity, which is not reversed by weight loss.

Similar impairments in metabolism were found for $CD8^+$ T cells. Prior to secondary infection, OCR was not different

**Table 1. Obese and Weight Loss Mice Have Greater Metabolic Change Following PR8 Influenza Infection**

| Day 0 Versus Day 7 Metabolic Comparison | | Fold Difference | Adjusted *P* Value | Fold Difference Relative to Always Lean |
|---|---|---|---|---|
| $CD4^+$ T cell basal OCR | Always lean | 0.33 | .7869 | … |
| | Always obese | **1.41** | **<.0001** | 3.21 |
| | Weight loss | **1.45** | **.0001** | 3.33 |
| $CD4^+$ T cell basal ECAR | Always lean | **−0.42** | **.0106** | … |
| | Always obese | **−0.71** | **<.0001** | 0.69 |
| | Weight loss | **−0.68** | **<.0001** | 0.62 |
| $CD4^+$ T cell OCR:ECAR | Always lean | **1.47** | **.0217** | … |
| | Always obese | **7.32** | **<.0001** | 3.98 |
| | Weight loss | **6.62** | **<.0001** | 3.50 |
| $CD8^+$ T cell basal OCR | Always lean | **1.22** | **.0163** | … |
| | Always obese | **2.20** | **<.0001** | 0.80 |
| | Weight loss | **1.73** | **<.0001** | 0.42 |
| $CD8^+$ T cell basal ECAR | Always lean | −0.16 | .6642 | … |
| | Always obese | **−0.67** | **<.0001** | 3.19 |
| | Weight loss | **−0.59** | **.0001** | 2.68 |
| $CD8^+$ T cell OCR:ECAR | Always lean | **1.93** | **.0379** | … |
| | Always obese | **8.42** | **<.0001** | 3.35 |
| | Weight loss | **5.74** | **<.0001** | 1.97 |

Metabolic differences in splenic $CD4^+$ and $CD8^+$ T cell basal OCR, basal ECAR, and OCR:ECAR ratio due to PR8 infection were determined. Fold differences from day 0 to day 7 in always lean (n = 10), always obese (n = 10), and weight loss (n = 10) mice were compared using 2-way ANOVA with Tukey multiple comparisons. Fold differences from day 0 to day 7 were compared between groups relative to fold difference in the always lean group. Bold indicates significant fold change from day 0 to day 7.

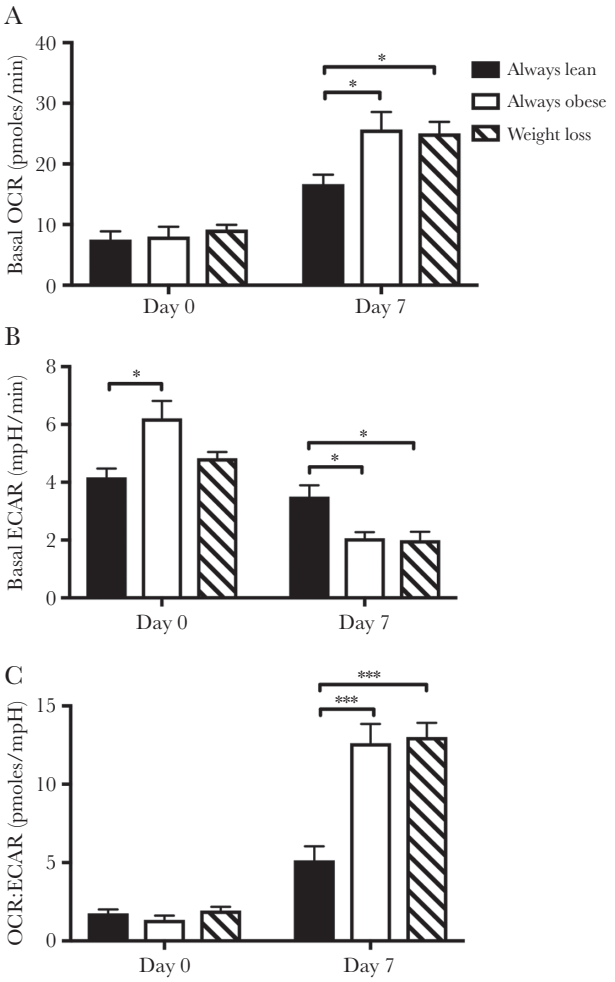


**Figure 6.** Altered CD8$^+$ T-cell metabolism is not reversed by weight loss. Spleens harvested from mice were used for CD8$^+$ T-cell isolation by negative selection magnetic bead separation. Cells were plated at 150 000 cells per well, and extracellular flux analysis was performed using the Seahorse XFe96 Flux analyzer at 37°C. *A*, Basal oxygen consumption rate (OCR) as a surrogate for mitochondrial respiration, (*B*) basal extracellular acidification rate (ECAR) as a surrogate for glycolysis, and (*C*) the ratio of oxidation to glycolysis (OCR:ECAR) were determined in CD8$^+$ T cells at day 0 and 7 after PR8 infection in always lean, always obese, and weight loss mice (n = 5 per group). Data represent mean ± standard error of the mean. One-way ANOVA with Tukey multiple comparisons were used at day 0 and day 7 to compare differences between groups. *$P < .05$, **$P < .01$, ***$P < .001$.

among groups (Figure 6A). However, following secondary infection, compared with always lean mice, CD8$^+$ T cells from always obese and weight loss mice had significantly elevated OCR (Figure 6A). ECAR of CD8$^+$ T cells prior to infection was significantly elevated in always obese mice, however ECAR was lower in both always obese and weight loss mice at day 7 postinfection (Figure 6B). The change in ECAR between day 0 and day 7 was significantly lower in obese and weight loss mice compared to always lean mice; however, ECAR did not change between day 0 and day 7 for the always lean mice (Table 1).

Similar to what was seen in CD4$^+$ T cells, OCR:ECAR ratios among groups were not different in CD8$^+$ T cells at day 0. However at day 7 postinfection, OCR:ECAR ratios were significantly higher in always obese and weight loss mice (Figure 6C). These findings indicate that the metabolic state of both CD4$^+$ and CD8$^+$ T cells is determined by the systemic metabolic state at the time of the primary infection during memory generation, as subsequent weight loss did not reset T-cell metabolism from an obese phenotype to a lean phenotype.

## DISCUSSION

Each year, 3000 to 56 000 people in the United States die from influenza infection, with approximately 500 million obese individuals worldwide at heightened risk [28, 29]. Although vaccination provides the best protection against influenza infection, our laboratory has shown, compared with lean adults, influenza-specific memory T cells from obese adults have decreased activation and function in response to ex vivo influenza challenge [9, 10]. Strikingly, despite vaccination, obese adults are 2 times more likely to develop influenza or influenza-like illness [14].

Obesity is a systemic metabolic disease, characterized by elevated serum insulin and glucose levels, along with increased adipose tissue mass accompanied by an increase in immune cell infiltration into the SVF surrounding adipocytes. In our model of diet-induced obesity, obese mice developed hyperglycemia and hyperinsulinemia, increased visceral fat mass, and an increase in both CD4$^+$ and CD8$^+$ T cells into the SVF. Thus, obesity induced by diet in this model mirrors the metabolic dysfunction of obese adults [30].

For memory T cells to effectively participate against reinfection, they must be present at the site of the secondary infection. Memory T-cell subsets in the lungs of mice reinfected with influenza virus, compared with lean mice, obese mice had significantly lower percentages of CD4$^+$ and CD8$^+$ Tcm, Trm, and Treg cells and a lower percent of CD8$^+$ effector T cells. Because it is well established that the metabolic state of T cells dictates their function [31], we reasoned that the systemic metabolic alterations of obesity would also affect T-cell metabolism.

Even prior to infection, we found T cells from obese mice had altered cellular metabolism characterized by increased OCR and ECAR. However, at 7 days postinfection, both CD4$^+$ and CD8$^+$ T cells from obese mice dramatically increased OCR compared to lean mice, with increased OCR:ECAR ratio, demonstrating an important switch in T-cell metabolic phenotype in obesity.

We hypothesized that weight loss would restore function and/or T-cell metabolism in formerly obese mice. Several studies report that weight loss does not completely reverse the effects of weight gain, with metabolite and inflammation signatures persisting following weight loss [32, 33]. Here we found weight loss could reverse the systemic hyperinsulinemia and hyperglycemia seen in obesity and significantly reduce visceral adipose tissue mass. However, weight loss failed to reduce infiltration of T cells into adipose tissue and did not reverse the memory

T-cell dysfunction seen in response to influenza reinfection. Notably, weight loss also did not reverse the obesity-associated metabolic dysfunction, with $CD4^+$ and $CD8^+$ T-cell OCR and OCR:ECAR ratios remaining elevated at day 7 postinfection, equivalent to the levels seen in always obese mice.

Altogether, our results demonstrate obesity induces a unique T-cell metabolic program that is associated with T-cell dysfunction and is not reversed by weight loss. These findings suggest obesity-associated T-cell metabolic dysfunction as a mechanism for altered T-cell infiltration and function in obesity. These results have significant public health importance and impact. Compared with lean adults, influenza vaccination of obese adults is less protective against influenza and influenza-like illness and our data suggest that weight loss alone, while important for metabolic health, may be insufficient to restore immune dysfunction and vaccine efficacy. In light of these results, it is imperative further interventions and revisions of vaccine strategies be considered.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

## Notes

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