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Diet-Induced Obesity in Mice Reduces the Maintenance of Influenza-Specific CD8+ Memory T Cells[1,2]

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Abstract

Obesity has been associated with increasing the risk for type 2 diabetes and heart disease, but its influence on the immune response to viral infection is understudied. Memory T cells generated during a primary influenza infection are important for protection against subsequent influenza exposures. Previously, we have demonstrated that diet-induced obese (DIO) mice have increased morbidity and mortality following secondary influenza infection compared with lean mice. To determine whether the problem resided in a failure to maintain functional, influenza-specific $CD8^+$ memory T cells, male DIO and lean mice were infected with influenza X-31. At 84 d postinfection, DIO mice had a 10% reduction in memory T cell numbers. This reduction may have resulted from significantly reduced memory T cell expression of interleukin 2 receptor β (IL-2Rβ, CD122), but not IL-7 receptor α (CD127), which are both required for memory cell maintenance. Peripheral leptin resistance in the DIO mice may be a contributing factor to the impairment. Indeed, leptin receptor mRNA expression was significantly reduced in the lungs of obese mice, whereas suppressor of cytokine signaling (*Socs*)*1* and *Socs3* mRNA expression were increased. It is imperative to understand how the obese state alters memory T cells, because impairment in maintenance of functional memory responses has important implications for vaccine efficacy in an obese population. J. Nutr. 140: 1691–1697, 2010.

Introduction

Obesity is a major global public health problem. Although humans are fairly well adapted to periods of reduced food intake, they are poorly adapted to overnutrition (1). Obesity can lead to serious health consequences and, subsequently, increases in health care requirements and economic burden. Resulting from increased energy intake and decreased expenditure (2), obesity has been linked to numerous health problems and chronic diseases (3,4). These comorbidities associated with obesity have been attributed to hormonal and metabolic changes related to increases in adipose tissue mass (5,6). Obesity is an independent risk factor for cardiovascular disease, type 2 diabetes, hypertension, arthritis, sleep apnea, and cancer (reviewed in 7); however, its effects on susceptibility to infection are poorly understood. In a hospital setting, obese patients are more likely to develop secondary infections and obese individuals are at increased risk for community-related respiratory tract infections (8–11). Studies in diet-induced obese (DIO)[3] animal models have shown obese mice had lower levels of mitogen-induced interleukin (IL)-2, although interferon (IFN)-γ and IL-4 production increased (12). Additionally, obese mice have impaired dendritic cell (DC) function and altered T cell responsiveness (13–19). Recently, obese individuals have been found to be at a greater risk of morbidity and mortality from infection with pandemic novel influenza H1N1 strain (20,21). At this time, it is unclear how obesity can result in a greater risk from H1N1 infection.

Influenza is a highly contagious, seasonal respiratory illness caused by the influenza virus. In any given year, 5–15% of the world population is infected with influenza virus, resulting in 3–5 million cases of severe illness and 500,000 deaths from influenza and influenza-related complications (22,23). Because of the propensity of influenza virus surface proteins to change each year, an antibody-based vaccine may be effective for one year but not the next (24). However, memory T cells target internal influenza virus proteins, which have little variation year to year (25). Therefore, a vaccine that targets expanding memory T cells may be more effective than a traditional antibody-based vaccine.

Previously, we showed that diet-induced obesity in mice significantly reduced the memory T cell response to secondary viral challenge, resulting in increased morbidity and mortality (24). To understand how obesity contributes to a poor response to secondary viral challenge, we examined the ability of DIO mice to maintain influenza-specific memory $CD8^+$ T cells.

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[3] Abbreviations used: DC, dendritic cell; DIO, diet-induced obese; γ_c, gamma chain; IFN, interferon; IL, interleukin; IL-2Rβ, interleukin 2 receptor β; IL-15R, interleukin 15 receptor; Lepr, leptin receptor; p.i., postinfection; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; T_{CM}, central memory T cell; T_{EM}, effector memory T cell; TNFα, tumor necrosis factor-α.

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Materials and Methods

Animals. Weanling, male C57BL/6J mice were obtained from Jackson Laboratories and housed at the University of North Carolina Animal Facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Animals were housed 4/cage under pathogen-free/viral Ab-free conditions and maintained under protocols approved by the Institutional Animal Use and Care Committee. Mice were randomized to receive either a low-fat diet (Research Diets; D12329) (n = 40) or a high-fat/high-sucrose diet (D12331) (n = 40) for 20 wk. The dietary composition was previously described by Surwit et al. (25,26). Previous studies in our laboratory (27) and others (28,29) have confirmed that these diets result in significant diet-induced obesity in these mice.

Influenza viruses and infection. Mouse-adapted influenza virus strain X-31 (H3N2) was grown in the allantoic fluid of embryonated hen's eggs. Influenza X-31 is a mouse-adapted, sublethal recombinant influenza virus (30). The X-31 strain has been shown to produce memory T cells able to prevent a lethal infection with a secondary influenza infection (31). For infection, following 20 wk of the diets, lean and obese mice were anesthetized intraperitoneally with ketamine/xylazine and subsequently inoculated intranasally with 300 EID_{50} live X-31 virus in 30 μL sterile PBS. Mice were maintained on the diets. By d 33 postinfection (p.i.), no virus was detected in the lungs of either lean or obese mice, demonstrating clearance of the infection (data not shown).

Quantitation of lung mRNA cytokine levels. Lung and spleen samples were collected on d 33 and 84 p.i. and total RNA was isolated using the TRIzol method. RT was carried out with Superscript II First Strand Synthesis kit (Invitrogen) using oligo (dT) primers. Following previously described methods for quantitative real-time PCR (27), mRNA levels for murine IL-6 (*Il6*), and tumor necrosis factor (*Tnfa*) and *Gapdh* were determined using previously described primer/probe sets and mRNA levels for *Il15* (Mm00434210_m1), *Il7* (Mm00434291_m1), *IL2rb* (Mm00434264_m1), leptin receptor (*Lepr*; Mm00440181_m1), suppressor of cytokine signaling (*Socs1*; Mm00782550_s1), *Socs3* (Mm00545913_s1), and *Actb* (Mm01205647_g1) were determined using TaqMan Gene Expression assays (Applied Biosystems).

Isolation of cells from the lungs, spleen, and draining lymph node. As previously described (32), lungs from lean and obese mice were removed and digested in HBSS (with Ca and Mg) supplemented with 160 kU/L Collagenase type 1 (Worthington). Spleen and draining lymph node (mediastinal) cells were isolated in unsupplemented HBSS. Samples were processed into single-cell suspensions by mechanical agitation of a Stomacher (Seward) and strained through a 40-μm nylon filter. Cells were subjected to RBC lysis using ACK lysis buffer for 5 min at room temperature, washed, counted then analyzed by flow cytometry.

Flow cytometry. At least 1×10^6 cells were stained with fluorescein isothiocyanate-anti-CD44, Pacific Blue-anti-CD62L (eBioscience) and peridinin-chlorophyll-protein complex-anti-CD8α (BD Biosciences). $CD8^+$ T cells specific for the major epitope of the A/PR/8 nucleoprotein were identified using a phycoerythrin-labeled $D^bNP_{366–374}$ tetramer. Nonspecific tetramer staining was analyzed using an irrelevant tetramer toward herpes simplex virus. Samples were analyzed on a Cyan ADP flow cytometer (Beckman Coulter) and data were analyzed using FlowJo software (TreeStar).

Statistical analysis. Nonparametric data were analyzed using the Kruskal-Wallis test (α = 0.05). Normally distributed data were analyzed by 2-way ANOVA with diet and day p.i. as main effects. Student's t test was used for post-hoc comparisons between the dietary groups and the days p.i. Differences were considered significant at $P < 0.05$. Statistical analyses were performed using JMP Statistical Software (SAS Institute).

Results

DIO mice have significantly increased weight compared with lean controls. At d 33 p.i., DIO mice weighed ~20 g more (57.3 ± 0.5 g) than their lean counterparts (37.3 ± 0.8 g). At d 84 p.i., lean mice had gained ~5 g of weight from d 33; however, lean mice still weighed ~15 g less (43.9 ± 1.2 g) than their DIO counterparts (56.2 ± 1.3 g).

Influenza-specific central memory T cells increase over time in the lung, spleen. Following a primary influenza infection, a small population of influenza-specific central memory T (T_{CM}) cells remain in secondary lymphoid tissues, which includes the bronchiole-associated lymphoid tissue of the lungs. In the lungs of both lean and obese mice, percent (**Fig. 1***A*) but not number (Fig. 1*B*) of T_{CM} cells increased over time. The lean and obese groups did not differ. In contrast, although percentage of T_{CM} cells increased in the spleen of both groups over time (Fig. 1*C*), at d 84 p.i., obese mice had significantly more T_{CM} in the spleen compared with lean controls (Fig. 1*D*).

DIO mice maintain significantly reduced numbers of influenza-specific $CD8^+$ effector memory T cells in the lungs. Following primary influenza infection, a population of influenza-specific effector memory T (T_{EM}) cells is maintained in the lung to more rapidly respond to the next encounter with an influenza virus. At d 33 post X-31 infection, both lean and obese mice had populations of influenza-specific T_{EM} cells in the lung (**Fig. 2***A*). In both the lean and obese mice, population percent and number of T_{EM} cells declined over time; however, the DIO mice had a greater percent loss of T_{EM} cell numbers, resulting in a significant decrease in T_{EM} cell numbers in the lungs of obese mice compared with lean controls at d 84 p.i. (Fig. 2*B*,*C*). In lymph nodes and spleen, the number of T_{EM} decreased over time in both the lean and obese mice; however, the 2 dietary groups did not differ (data not shown).

CD122 (IL-2Rβ) expression is significantly reduced on influenza-specific T_{EM} cells in the lungs of obese mice. In both lean and obese mice, the percentage of T_{EM} cells expressing CD122 increased over time; however, at d 33 p.i., the percentage of T_{EM} cells expressing CD122 was significantly lower in the lungs of obese mice compared with lean controls (**Fig. 3***A*,*B*). In addition to reduced percentage of cells expressing CD122, mean

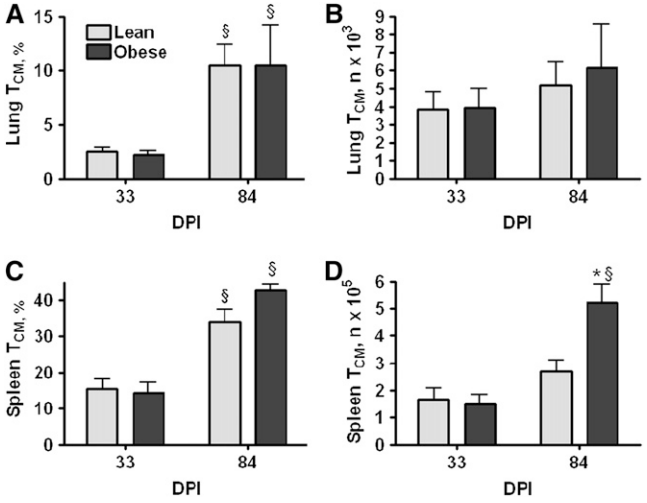

FIGURE 1 Percentage (*A*,*C*) and number (*B*,*D*) of influenza-specific T_{CM} in lung (*A*,*B*) and spleen (*C*,*D*) of lean and obese mice at d 33 and 84 p.i. T_{CM} ($CD8^+/D^bNP_{366–74}{}^+/CD44^+/CD62L^+$) were identified using flow cytometry. Data are means + SEM, n = 6. Symbols indicate differences ($P < 0.05$): * vs. lean; § vs. d 33.

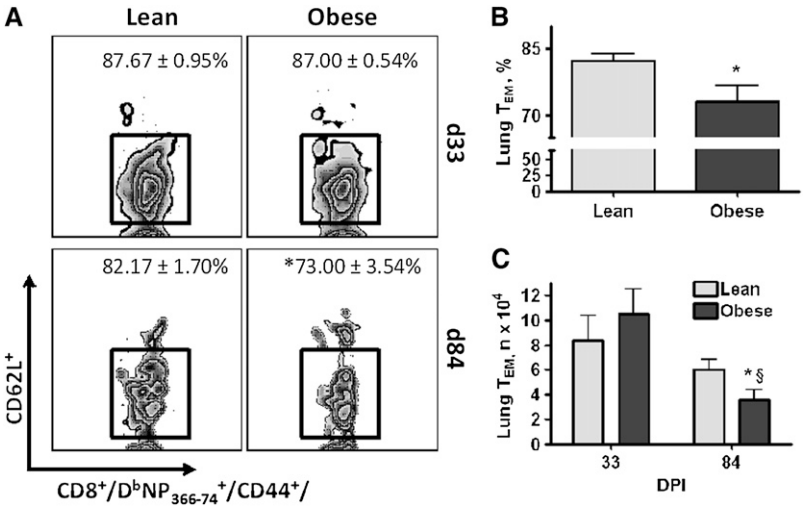

FIGURE 2 Percentage (*A,B*) and number (*C*) of influenza-specific CD8+ T_{EM} in lungs of lean and obese mice at d 33 and 84 p.i. T_{EM} ($CD8^+/D^bNP_{366–74}{}^+/CD44^+/CD62L^-$) were identified using flow cytometry. In *B*, data were obtained on d 84 p.i. Data are means + SEM, $n = 6$. Symbols indicate differences ($P < 0.05$): * vs. lean; § vs. d 33.

fluorescence intensity of CD122 was also significantly lower on T_{EM} cells in the lungs of obese mice (Fig. 3C). Lung CD122 mRNA expression did not differ between obese and lean mice (data not shown).

Obese mice have altered expression of Il15 *and Il7 mRNA in the lungs post primary infection.* IL-7 and IL-15 are the cytokines responsible for the homeostasis and survival of memory T cells. In the lungs of obese mice, *Il15* mRNA expression at d 84 p.i. was ~3 times greater than in lean controls (**Table 1**). In contrast, expression of *Il7* mRNA was 50% lower at d 33 in the lungs of obese mice compared with lean controls. Interestingly, in both lean and obese mice, expression of *Il7* mRNA significantly increased over time.

Leptin receptor expression is reduced and Socs1 *and Socs3 expression is increased in the lungs of obese mice.* In the lungs of obese mice, *Lepr* mRNA expression was significantly less than in lean mice at both d 33 and 84 p.i. *Lepr* expression increased in the lungs of lean mice by d 84 post X-31 infection; however, expression did not increase in the lungs of obese mice. Additionally, expression of *Socs1* and *Socs3* significantly increased in the lungs of obese mice at d 84 p.i. (Table 1).

The lungs of obese mice have significantly increased inflammation in the absence of viral infection. Following clearance of the X-31 infection at d 33 p.i., mRNA expression of *Tnfa* but not *Il6* significantly increased in the lungs of obese mice compared with lean controls. At d 84 p.i., mRNA expression of both *Tnfa* and *Il6* significantly increased (Table 1).

Discussion

A vaccine that promotes a robust memory B cell antibody-based response to the surface proteins of 1 strain of influenza could be ineffective for a strain encountered the next season expressing different surface proteins (33). However, there are several internal viral proteins that are highly conserved among influenza viruses and therefore do not experience the mutations seen with the external proteins. Although these internal proteins do not generate an effective antibody response because of their lack of accessibility to antibodies, the cell-mediated immune response can recognize these proteins. Therefore, memory T cells generated during a primary influenza infection can target these internal proteins common to influenza strains, making them effective against encounters with heterologous virus stains. The ability to generate functional memory T cells, either during a primary infection or by vaccination, has proven to be protective against potentially lethal influenza strains exhibiting completely different surface antigens (34). Following viral clearance, contraction of activated $CD8^+$ T cells results in influenza-specific memory cells representing ~5–10% of effector $CD8^+$ T cells found at the peak of expansion during primary infection (35,36).These long-lived memory cells can rapidly respond to a secondary infection with influenza virus.

Although the exact definitions are still controversial, memory T cells established after infection can be divided into 2 subsets: T_{CM}, which preferentially localize to the lymphoid tissues, and T_{EM}, which are found mainly in peripheral sites of infection (37,38). Numbers of T_{CM} in lymphoid sites appear to remain relatively constant, if not slightly increased, whereas the number of T_{EM} at peripheral sites steadily decreases in the months after pathogen clearance. Although both lean and obese mice demonstrated the expected increase in percent T_{CM} and decrease in T_{EM}, the T_{EM} in the lungs of the obese animals declined at a significantly faster pace, resulting in a 10% reduction in the numbers of T_{EM} present compared with the lean controls. This reduction in the numbers of influenza-specific T_{EM} in the lungs of obese mice has major health implications, because the protective capacity from a secondary infection can be directly linked to the number of pathogen-specific memory T cells present in the tissue prior to secondary infection (39). Therefore, as we have already observed a decrease in protective capacity in influenza-specific memory T cells in obese mice (24), a reduction in numbers of these cells could decrease protection even further.

Memory cells persist under normal conditions by undergoing intermittent cell division, called bystander proliferation, about once every 2 wk (40). Originally, the maintenance and survival of memory T cells was thought to be mediated by signals arising from contact with depots of persistent antigens; however, memory cells have been shown to survive not only in the absence of antigen but also in the absence of major histocompatibility complex molecules (41,42). Survival of memory cells now appears to be maintained by members of the common gamma chain (γ_c) family of cytokines, IL-15, and IL-7.

IL-15 is essential for the regulation of slow, intermittent basal homeostatic proliferation of antigen-specific $CD8^+$ memory T cells (43). Viral challenge of $IL15^{-/-}$ and $IL\text{-}15R\alpha^{-/-}$ mice show that IL-15 is dispensable for the generation of antigen-specific

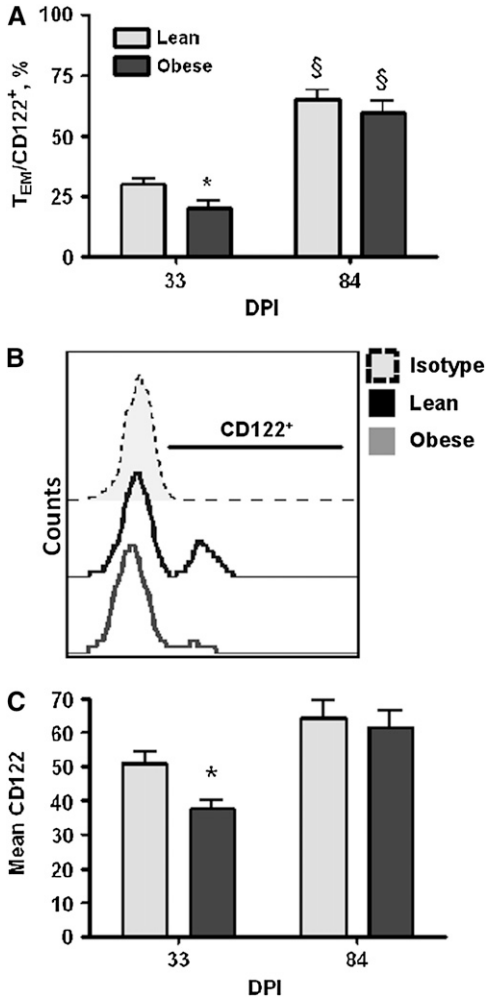

FIGURE 3 Percentage (*A*), count (*B*), and mean fluorescence intensity (*C*) of CD122 expression on influenza-specific T_{EM} cells in lungs of lean and obese mice at d 33 and 84 p.i. CD122 expression on T_{EM} cells ($CD8^+/D^bNP_{366–74}{}^+/CD44^+/CD62L^-$) was identified using flow cytometry. In *B*, data were obtained on d 33 p.i. Data are means + SEM, $n = 6$. Symbols indicate differences ($P < 0.05$): * vs. lean; § vs. d 33.

TABLE 1 Lung mRNA expression in lean and obese mice following influenza infection[1]

	Lean, d 33 p.i.	Obese, d 33 p.i.	Lean, d 84 p.i.	Obese, d 84 p.i.
		Fold of control[2]		
Il15	1.00 ± 0.62	0.73 ± 0.45	6.46 ± 3.25	28.9 ± 9.39[a,b]
Il7	1.00 ± 0.43	0.20 ± 0.08[a]	4.11 ± 1.94[b]	3.13 ± 1.17[b]
Lepr	1.00 ± 0.33	0.35 ± 0.10[a]	2.69 ± 0.78[b]	1.53 ± 0.49
Socs1	1.00 ± 0.26	1.55 ± 0.31	1.61 ± 0.23	3.87 ± 0.86[a,b]
Socs3	1.00 ± 0.27	1.40 ± 0.73	2.18 ± 0.97	6.72 ± 2.41[a,b]
Tnfa	1.00 ± 0.50	3.23 ± 0.86[a]	0.63 ± 0.09	4.53 ± 2.16[a]
Il6	1.00 ± 0.56	4.96 ± 2.45	1.64 ± 1.18	8.82 ± 3.26[a]

[1] Values are means ± SEM, $n = 5–6$. Letters indicate differences ($P < 0.05$): [a] vs. lean; [b] vs. d 33 pi.
[2] Data were normalized to β-actin and expressed relative to lean, d 33 p.i.

$CD8^+$ memory T cells; however, the memory T cell pool decreased over time (44,45), as bystander proliferation fails to occur. Wild-type memory $CD8^+$ T cells transferred into IL-$15^{-/-}$ mice do not proliferate and disappear rapidly (46). Overexpression of IL-15 increased the numbers of memory $CD8^+$ cells (47,48). In the lean mice, *Il15* mRNA expression was maintained at a constant level; however, expression of mRNA for *Il15* in the obese mice was ~30 times greater at d 84 p.i. compared d 33. This increase in *Il15* mRNA did not result in increased T_{EM}. In fact, fewer T_{EM} were found in the obese lung.

Several possibilities may explain this finding. For example, a reduction in IL-15R may result in decreased T_{EM} even under conditions of increased IL-15 expression. The IL-15R consists of high-affinity IL-15Rα, IL-2/15Rβ (CD122), and γ_c subchains that form a heterotrimeric receptor complex. Memory CD8+ T cells express high levels of CD122. Obese, but not lean, Zucker rats have significantly decreased expression of mRNA for the γ_c and CD122 subunits of the IL-15 receptor whereas IL-15Rα remained unchanged (49). Whereas the mRNA expression of *Il2Rb* and *Il15ra* in the lungs of lean and obese mice did not differ (data not shown), the numbers of T_{EM} expressing CD122 were significantly reduced in the lungs of DIO mice 33 d post primary infection. In addition, the mean fluorescence intensity of CD122 was also significantly reduced on T_{EM} expressing the receptor, indicating a decreased amount of expression on these cells. However, by d 84, CD122 expression did not differ between lean and obese mice. Therefore, alterations in IL-15R levels likely do not account for the decrease in T_{EM}. In addition, it should be noted that our study measured mRNA levels for IL-15, which may not accurately reflect protein levels.

A second possibility for decreased T_{EM} in the presence of increased IL-15 levels is a blockade of IL-15 action due to leptin resistance. Elevated levels of leptin in the obese state results in attenuated central leptin signaling, with likely decreased signaling in the periphery as well. Leptin, IL-7, and IL-15 share structural homology and signal through the Janus Kinase-signal transducer and activator of transcription (STAT) pathway. IL-15 has been found to play a role in lipid metabolism, fat deposition, and insulin sensitivity. Recently, IL-15 administration to healthy lean rodents was found to reduce white adipose deposition without changing food consumption (50,51). Mice overexpressing IL-15 exhibit reduced body fat and increased resistance to DIO (51–53). IL-15 administration to obese leptin-deficient (*ob/ob*) mice, but not *Lepr*-deficient obese (*fa/fa*) rats, resulted in decreased fat deposition. Thus, leptin signaling appears to be very important for the function of IL-15. In the lungs of obese mice, *Lepr* mRNA expression was significantly decreased at d 33 p.i. and did not increase over time as in lean controls, suggesting that leptin signaling was likely impaired.

Besides *Il15* mRNA expression, the obesigenic state may affect other aspects of IL-15 function. Trans-presentation of IL-15 by antigen-presenting cells, particularly DC, has been shown to be very important for memory T cell maintenance. IL-15 is bound to IL-15Rα on the surface of the DC and stimulates neighboring cells through the CD122/γ_c complex (50–53). Because previous studies in our laboratory (32) and others (54,55) have shown DC functionality is affected by obesity and alterations in leptin signaling, decreased IL-15 trans-presentation by DC could contribute to the decreased memory cell maintenance in the lungs of obese mice.

Characteristic of leptin resistance, mRNA expression of *Socs1* and *Socs3*, potent inhibitors of Janus Kinase-STAT signaling, were significantly increased in the lungs of obese mice. The participation of SOCS proteins, particularly SOCS-3 but also SOCS-1, as negative-feedback regulators of leptin signaling has been suggested as a causal factor for central leptin resistance (56–58). Indeed, SOCS-3 deficiency elevates leptin sensitivity and confers resistance to diet-induced obesity (59,60).

Both SOCS-1 and SOCS-3 can inhibit Lepr signaling (61,62). SOCS proteins are induced upon cytokine stimulation and attenuate signaling by various cytokine receptors, allowing possible cross-regulation among several cytokine systems. For example, SOCS-3 is induced by IL-2, IL-6, and leptin and can inhibit leptin, IL-2, IL-4, IL-6, IFNα/β, and IFNγ signaling (63–67). Although SOCS-3 has not yet been directly linked to IL-15 signaling, it has been observed that SOCS-1 expression can directly regulate IL-15–driven homeostatic proliferation and SOCS-$1^{-/-}$ mice accumulate $CD44^+/CD8^+$ memory phenotype T cells, which express elevated levels of CD122 (68,69). We observed a decrease in the numbers of $CD44^+/CD8^+$ memory T cells in obese mice despite an increase in *Il15* mRNA expression. Therefore, the increased SOCS expression in the lungs due to leptin resistance could be responsible for the failure of IL-15 to signal, thereby preventing the bystander proliferation required for memory T cell maintenance and resulting in a significantly reduced, lung-resident T_{EM} population over time.

Obesity has also been associated with chronic inflammation and, indeed, significant increases in *Il6* and tumor necrosis factor (TNF)-α (*Tnf*α) mRNA expression were seen in the lungs of obese mice at d 33 and d 84 p.i. Although increased adipose tissue and serum levels of TNFα and IL-6 have been reported previously (5,70–74), our work demonstrates that chronic inflammation can also occur in the lungs of obese mice. This increased inflammation has been associated with leptin resistance, because these cytokines could contribute to or be the result of upregulation of SOCS proteins (75). Indeed, *Socs1* mRNA expression has been found to attenuate IFN-dependent signaling pathways as well as insulin signaling (76,77). Inability to regulate signaling in these pathways in obese mice could be important for the generation of chronic inflammation and altered type I IFN (IFNI) responses seen in the DIO mice (24,27). IFNI appears to be very important for the production of the basal level of IL-15. IFNI receptor-deficient mice have one-half as many $CD122^{hi}$ memory cells compared with normal C57BL/6 controls. This is even more pronounced in STAT1-deficient mice, which are unresponsive to both IFNI and IFNγ (42).

Although maintenance of memory T cell populations is regulated by IL-15 and IL-7, other factors could contribute to the reduction in the numbers of influenza-specific T_{EM} in the lungs of obese mice over time. Both IL-15 and IL-7 increase the resistance of memory T cells to apoptosis, allowing them to persist for long periods of time. Interestingly, leptin signaling can also be attributed to reduction of T cell apoptosis, because leptin treatment has been found to prevent the decline of T cell numbers in the fasted state (78). If memory T cells in the lungs of obese mice are leptin resistant, then memory cells in the obesigenic environment would be at increased sensitivity to inflammation-induced apoptosis.

DIO mice are able to generate influenza-specific memory $CD8^+$ T cells; however, the functional capability of these cells (24) and their ability to be maintained in the lung is significantly reduced. The understanding of memory T cell maintenance is still in its infancy; however, it is clear that IL-15 and IL-7 and their subsequent signals are important for maintaining the memory cell population over time. Our study has shown that diet-induced obesity can affect the maintenance of influenza-specific memory T cell populations in the lungs. This decrease in memory T cell numbers may be due to peripheral leptin resistance in the obesigenic lung microenvironment affecting IL-15 function. Overall, decreased numbers of T_{EM} over time, coupled with the decreased functional capability of these cells, suggests that obesity presents an even greater risk to increased morbidity and mortality from infection with a heterologous pandemic strain. Indeed, vaccine strategies that promote the generation of memory T cells may be less effective in an obese population.

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