Lymphatic Function Regulates Contact Hypersensitivity Dermatitis in Obesity

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Obesity is a major risk factor for inflammatory dermatologic diseases, including atopic dermatitis and psoriasis. In addition, recent studies have shown that obesity impairs lymphatic function. As the lymphatic system is a critical regulator of inflammatory reactions, we tested the hypothesis that obesity-induced lymphatic dysfunction is a key regulator of cutaneous hypersensitivity reactions in obese mice. We found that obese mice have impaired lymphatic function, characterized by leaky capillary lymphatics and decreased collecting vessel pumping capacity. In addition, obese mice displayed heightened dermatitis responses to inflammatory skin stimuli, resulting in both higher peak inflammation and a delayed clearance of inflammatory responses. Injection of recombinant vascular endothelial growth factor-C remarkably increased lymphangiogenesis, lymphatic function, and lymphatic endothelial cell expression of chemokine (C–C motif) ligand 21, while decreasing inflammation and expression of inducible nitrous oxide synthase. These changes resulted in considerably decreased dermatitis responses in both lean and obese mice. Taken together, our findings suggest that obesity-induced changes in the lymphatic system result in an amplified and a prolonged inflammatory response.

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INTRODUCTION

It is estimated that more than one-third of US adults are obese, with the incidence rapidly rising in adult and pediatric populations (Ogden *et al.*, 2014). Obesity is associated with a wide variety of pathologies, including coronary artery disease, insulin resistance, diabetes, and malignancy (Garfinkel, 1985; Mokdad *et al.*, 2003; Lavie *et al.*, 2009; Glass and Olefsky, 2012). In addition, obesity has a profound impact on a variety of dermatologic diseases, including psoriasis and atopic dermatitis in both children and adults (Marino *et al.*, 2004; Silverberg *et al.*, 2011, 2012).

Chronic inflammation is a major mechanism regulating pathological changes in obesity. For example, visceral adipose deposition in obese individuals is associated with chronic infiltration of activated T cells and macrophages. These changes result in increased local and systemic expression of a multitude of inflammatory cytokines, including tumor necrosis factor-α, IL-6, IL-1β, chemokine (C–C motif) ligand 2 (CCL2), and others (Gregor and Hotamisligil, 2011; Lumeng and Saltiel, 2011). These inflammatory changes have crucial roles in the development of metabolic syndrome, insulin resistance, tumor metastasis, and cardiovascular disease (Mokdad *et al.*, 2003; Lavie *et al.*, 2009; Glass and Olefsky, 2012; Perez-Hernandez *et al.*, 2014). Similarly, previous studies have shown that obese patients have increased subcutaneous tissue inflammation, and that these pathological changes contribute to the development of dermatological disorders (Hamminga *et al.*, 2006; Gerdes *et al.*, 2011). However, although it is clear that inflammation is a key regulator of pathological outcomes in obesity, the effects of these changes on dermatological diseases and the regulation of inflammatory responses in obesity remain poorly understood.

Recent studies have shown that obesity impairs lymphatic function both in mice and in humans (Lim *et al.*, 2009; Arngrim *et al.*, 2013; Lim *et al.*, 2013; Weitman *et al.*, 2013; Savetsky *et al.*, 2014). For example, our group has reported that obese mice have decreased ability to transport interstitial fluid via cutaneous lymphatics and have remarkably decreased trafficking of antigen-presenting cells to regional lymph nodes (Weitman *et al.*, 2013). Furthermore, Rutkowski *et al.* (2010) demonstrated that adipose tissue accumulation

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Abbreviations: CCL, chemokine (C–C motif) ligand; DNFB, 1-fluoro-2,4-dinitrobenzene; HFD, high-fat diet; iNOS, inducible nitrous oxide synthase; LYVE-1^{+}*, lymphatic vessel endothelium hyaluronan receptor 1; NIR, near-infrared; rhVEGF-C, recombinant human VEGF-C; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor*

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reduces interstitial fluid transport by lowering hydraulic conductivity. Other investigators have shown that obese mice have impaired lymphatic collecting vessel pumping function (Blum *et al.*, 2014). These findings are supported by clinical studies demonstrating that obese patients have decreased clearance of macromolecules from fat depots when compared with normal controls, and that massively obese patients spontaneously develop lymphedema (a condition in which impaired lymphatic drainage function results in chronic limb swelling) (Greene *et al.*, 2012; Arngrim *et al.*, 2013). The relationship between obesity and the lymphatic system appears to be bidirectional, as previous studies have shown that mice with congenital lymphatic defects resulting from *Prox-1* haploinsufficiency go on to develop adult onset obesity (Harvey *et al.*, 2005). As the lymphatic system is a critical physiologic regulator of inflammation and immune responses, obesity-induced lymphatic dysfunction may act in a feed-forward manner to amplify the pathological consequences of obesity in end organs. This hypothesis is supported by previous studies demonstrating that improving lymphatic function with exogenous delivery of lymphangiogenic cytokines, such as vascular endothelial growth factor (VEGF)-C, decreases acute/chronic skin inflammation in lean mice (Kataru *et al.*, 2009; Huggenberger *et al.*, 2010). Thus, improving lymphatic function in obese patients may serve as a means of mitigating the pathology of cutaneous disorders associated with obesity.

In the current study, we investigated the role of the lymphatic system in regulating dermatitis in obesity. We found that obese mice have impaired lymphatic function and heightened dermatitis responses to inflammatory skin stimuli. Obese mice had both higher peak inflammation and a delayed clearance of inflammatory responses. Increasing lymphatic function by injection of recombinant human VEGF-C (rhVEGF-C) remarkably decreased dermatitis responses in obese mice, leading to decreased peak inflammation and an increased rate of its clearance. Taken together, our findings suggest that obesity-induced changes in the lymphatic system result in an amplified and a prolonged inflammatory response in the skin.

RESULTS

Obese mice have impaired lymphatic function at baseline

As expected, feeding male C57BL/6J mice a high-fat diet (HFD) for 10–12 weeks resulted in a significant increase in body weight and subcutaneous adipose deposition as reflected by increased perilipin-positive cell accumulation and ear thickness when compared with mice fed a normal chow diet (Figure 1a–c; $P<0.01$ for both). Consistent with our previous studies, immunofluorescence analysis of ear skin demonstrated significant baseline increases in the number of $CD45^+$ and $CD3^+$ cells in obese mice as compared with lean controls (Figure 1d and e, and Supplementary Figure S1a and b online; $P<0.01$ for all) (Weitman *et al.*, 2013; Savetsky *et al.*, 2014). In addition, obese mice had significantly fewer subcutaneous lymphatic vessel endothelium hyaluronan receptor 1 $(LYVE\text{-}1)^+$ lymphatic vessels as compared with controls (Figure 1d and f; $P<0.01$). Lymphangiography using subdermal Evans blue dye injection demonstrated the presence of leaky capillary lymphatics (white arrows) in obese mice as compared with lean controls (Figure 1g; upper panel). These findings were confirmed with functional lymphatic vessel staining using FITC fluorescently conjugated tomato lectin in whole-mounted specimens stained for α-smooth muscle actin and LYVE-1. Obese animals had significantly less uptake (yellow arrows) and more extravasation of peripherally injected tomato lectin in the ear (white arrow and outlined region) as compared with controls (Figure 1g; lower panel). Furthermore, analysis of collecting lymphatic pumping frequency in the hind limb and tail demonstrated a 2- to 3-fold decrease in packet frequency (visualized using near-infrared (NIR) imaging) in obese mice as compared with controls (Figure 1h and Supplementary Movies S1 online; $P<0.05$ and $P<0.01$ for the tail and hind limb, respectively). Finally, consistent with our observation that obese mice have impaired lymphatic function, we found that obese mice had significantly decreased systemic uptake of peripherally injected macromolecules (FITC–dextran) as compared with controls (Supplementary Figure S2b online; $P<0.05$).

Obese mice have normal antibody production but impaired T-cell recall

Given the decreased lymphatic function in obesity, we next investigated basal immune responses in obese mice. Obese mice had a normal ability to produce antibody responses (anti-OVA IgG_1 titers) as compared with controls (Supplementary Figure S3a online; $P=$NS). In contrast, T cells collected from obese mice and stimulated *ex vivo* using OVA produced significantly less IFNγ and IL-4 as compared with controls, indicating an impairment in T-cell recall (Supplementary Figure S3b and c online; $P<0.01$ and $P<0.05$, respectively). Impaired T-cell recall in obese mice was confirmed with *ex vivo* stimulation with 2,4-dinitrobenzenesulfonic acid after *in-vivo* sensitization with 1-fluoro-2,4-dinitrobenzene (DNFB) demonstrated significantly less IFNγ production as compared with controls (Supplementary Figure S3d online; $P<0.05$).

Obese mice have increased contact hypersensitivity

On DNFB challenge, obese mice had increased ear erythema, swelling, ear thickness, and epidermal thickness at days 3 and 8 as compared with controls, indicating a more severe inflammatory response (Figure 2a–c and Supplementary Figure S4a online; $P<0.01$ and $P<0.05$, respectively). Flow cytometric analysis of ear tissues collected from obese mice demonstrated increased numbers of activated T cells ($CD4^+$/$CD69^+$) (Figure 2d). This correlated with analysis of IFNγ protein levels in ear tissues and serum, which showed increased tissue and serum levels of IFNγ in obese mice (Figure 2e and f; $P<0.01$ for all). Although lean animals had a 3-fold increase in tissue IFNγ levels, obese mice tissues had a more than 8-fold increase 3 days after DNFB challenge. Similarly, although lean mice had only a mild increase in serum IFNγ levels (~30%), we noted an almost 2-fold increase in serum levels in obese mice.

Flow cytometry of ear tissues was also performed to analyze T-cell subtypes in lean and obese mice after DNFB challenge.