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Authors

Hu, Lizhi
Mauro, Theodora M
Dang, Erle
et al.

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Epidermal Dysfunction Leads to an Age-Associated Increase in Levels of Serum Inflammatory Cytokines

Lizhi Hu[1,5], Theodora M. Mauro[2,5], Erle Dang[2,3,5], George Man[2], Jing Zhang[1], Dale Lee[2], Gang Wang[3], Kenneth R. Feingold[2,4], Peter M. Elias[2] and Mao-Qiang Man[2]

Even though elderly populations lack visible or other clinical signs of inflammation, their serum cytokine and C-reactive protein levels typically are elevated. However, the origin of age-associated systemic inflammation is unknown. Our previous studies showed that abnormalities in epidermal function provoke cutaneous inflammation, and because intrinsically aged skin displays compromised permeability barrier homeostasis and reduced stratum corneum hydration, we hypothesized here that epidermal dysfunction could contribute to the elevations in serum cytokines in the elderly. Our results show first that acute disruption of the epidermal permeability barrier in young mice leads not only to a rapid increase in cutaneous cytokine mRNA expression but also an increase in serum cytokine levels. Second, cytokine levels in both the skin and serum increase in otherwise normal, aged mice (>12 months). Third, expression of tumor necrosis factor-α and amyloid A mRNA levels increased in the epidermis, but not in the liver, in parallel with a significant elevation in serum levels of cytokines. Fourth, disruption of the permeability barrier induced similar elevations in epidermal and serum cytokine levels in normal and athymic mice, suggesting that T cells play a negligible role in the elevations in cutaneous and serum inflammatory cytokines induced by epidermal dysfunction. Fifth, correction of epidermal function significantly reduced cytokine levels not only in the skin but also in the serum of aged mice. Together, these results indicate that the sustained abnormalities in epidermal function in chronologically aged skin contribute to the elevated serum levels of inflammatory cytokines, potentially predisposing the elderly to the subsequent development or exacerbation of chronic inflammatory disorders.

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INTRODUCTION

Although most aged humans display no clinical indications of inflammation, they typically have elevated serum cytokine levels (Kim et al., 2011; Mariani et al., 2006). Although an increase in adipose tissue that commonly occurs with aging is well known to increase serum cytokine levels (Surmi and Hasty, 2008), whether other organs, such as the skin, might also contribute to these elevations is unknown. However, for a single organ with unapparent inflammation to cause systemic inflammation, it should account for a substantial portion of body size. In this respect, the skin qualifies, because it accounts for 15% or more of total body weight (Kanitakis, 2002).

[1]*School of Basic Medical Sciences, Tianjin Medical University, Tianjin, People's Republic of China;* [2]*Dermatology Services, Veterans Affair Medical Center and University of California–San Francisco, San Francisco, California, USA;* [3]*Department of Dermatology, Xijing Hospital, Fourth Military Medical University, Xi'an, People's Republic of China; and* [4]*Metabolism, Veterans Affair Medical Center and University of California–San Francisco, San Francisco, California, USA*

[5]*These authors contributed equally to this work.*

Correspondence: Mao-Qiang Man, Dermatology Service (190), 4150 Clement Street, San Francisco, California 94121, USA. E-mail: mqman@hotmail.com or Lizhi Hu, School of Basic Medical Sciences, Tianjin Medical University, 22 Qixiangtai Rd, Heping Qu, China, 300070. E-mail: lizhihuleopard@yahoo.com

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Previous studies from our group and others have shown that either acute or chronic abrogation of epidermal permeability barrier function stimulates epidermal cytokine and chemokine production, inflammatory cell infiltration, and Langerhans cell maturation and proliferation (Katoh et al., 1997; Lin et al., 2013; Nishijima et al., 1997; Onoue et al., 2009; Proksch et al., 1996; Tsai et al., 1994; Wood et al., 1992, 1994, 1997) and also predisposes aged skin to *Staphylococcus aureus* colonization (Wanke et al., 2013). Finally, it should be noted that prolonged reductions in stratum corneum hydration also induce or aggravate cutaneous inflammation, independent of barrier disruption (Ashida and Denda, 2003; Ashida et al., 2001; Denda et al., 1998).

Because previous studies from our group and others showed that chronologically aged skin displays both compromised permeability homeostasis and reduced stratum corneum hydration, both of which increase cutaneous cytokine production (Choi et al., 2007; Ghadially et al., 1995; Kikuchi et al., 2003; Man et al., 2009, 2015a; Tsai et al., 1994; Wood et al., 1992, 1997), and increased susceptibility to infections (Laube, 2004), we hypothesized that epidermal dysfunction-induced cutaneous inflammation could lead to elevations in serum cytokines. In support of our hypothesis, both psoriasis and atopic dermatitis display prominent abnormalities in epidermal function, which has been proposed to drive the inflammation of these disorders (Elias and Steinhoff, 2008; Man et al., 2015b; Sano, 2015), and elevated serum cytokine levels correlate with the severity

of these disorders (Arican et al., 2005; Jacob et al., 2003; Yamamoto et al., 2013; Yoshizawa et al., 2002). However, the origin of serum cytokines in these two inflammatory dermatoses is controversial, because these disorders are considered to be T-cell–mediated immune diseases. In this study, we assessed whether the epidermal dysfunction, which inevitably accompanies intrinsically aged skin, could account for or contribute to the elevated levels of serum cytokines in the elderly. We present evidence here that age-related epidermal dysfunction leads to elevations in serum inflammatory cytokines in aged mice; and conversely, that correction of epidermal functional abnormalities reduces both cutaneous and serum cytokine levels. Together, these studies raise the intriguing possibility that epidermal functional abnormalities could contribute to the development of certain chronic age-associated systemic disorders.

RESULTS

Acute disruption of the epidermal permeability barrier increases cutaneous and serum inflammatory cytokines in young mice

Prior studies from our group and others have shown that disruption of the epidermal permeability barrier increases epidermal cytokine production, eventually inducing dermal inflammation in murine models (Lin et al., 2013; Proksch et al., 1996; Tsai et al., 1994; Wood et al., 1992, 1994, 1997). However, whether acute barrier disruption elevates serum levels of inflammatory cytokines is unknown. To test our hypothesis that epidermal dysfunction can induce an increase in serum inflammatory cytokine levels, we first determined whether acute abrogation of epidermal permeability barrier function increases serum levels of cytokines in young mice. As seen in Figure 1a, serum levels of IL-1α, IL-1β, IL-6, and tumor necrosis factor (TNF)-α increased significantly 3 hours after acute barrier abrogation induced by repeated tape-stripping. In a separate experiment, we assessed whether the serum levels of serum amyloid A, a well-accepted marker of acute systemic inflammation, changes after barrier disruption. Our results show that the serum levels of amyloid A dramatically increased after acute barrier disruption (by over 200% and 100% at 2 and 6 hours, respectively; $P < 0.001$ for both time points).

Because our previous studies focused on the influence of epidermal permeability disruption on epidermal cytokine production (Tsai et al., 1994; Wood et al., 1992, 1994, 1997), we next determined whether disruption of epidermal permeability barrier up-regulates cytokine expression not only in the epidermis, but also in the dermis. In agreement with our previous findings, barrier disruption induced a significant increase in the mRNA levels of IL-1α, IL-1β, IL-17F, IL-19, IL-22, IL-23, and TNF-α in the epidermis, and further, it also up-regulated expression of these cytokine mRNAs in the dermis (red blocks in Figure 1b). In contrast, epidermal mRNA levels of certain T helper type 2 cytokines, that is, IL-4 and IL-5, as well as IL-10, either did not change or decreased (Figure 1b), and mRNA levels of the T-cell chemoattractant CXCL-9 decreased in both the dermis and epidermis after barrier disruption (Figure 1b). Taken together, these results show that acute abrogation of the epidermal permeability barrier function induces an increase in inflammatory cytokines in both the skin and serum.

Aged mice display increased levels of cutaneous and serum inflammatory cytokines

Both aged human and mouse epidermis exhibits functional abnormalities, including reduced stratum corneum hydration, and compromised epidermal permeability barrier homeostasis, which first appears at age 50 years or older in humans (12–15 months in mice) (Choi et al., 2007; Ghadially et al., 1995). To examine whether age-associated epidermal dysfunction is accompanied by elevations in cutaneous and serum inflammatory cytokines, we next assessed changes in proinflammatory cytokine levels in both the skin and serum of otherwise normal-appearing, aged mice under basal condition. As shown in Figure 2a, the expression levels of mRNA for IL-1α, IL-19, IL-23, transforming growth factor-β, TNF-α, and CXCL-9 increased significantly in both the dermis and epidermis of 12-month-old versus 8-week-old mice (red blocks in Figure 2a). Consistent with these increases in mRNA levels, epidermal TNF-α protein levels also increased 5-fold in aged mice versus young mice (0.87 ± 0.16 in young vs. 5.33 ± 0.56 in aged; $P = 0.0016$). In contrast, the mRNA levels of epidermal IFN-γ, a cytokine primarily produced by natural killer T cells, diminished in the aged mice (Figure 2a). Similarly, the levels of dermal CXCL-2, a chemokine predominantly secreted by monocytes and macrophages, also decreased in aged mice. To determine whether aged skin displays increased infiltration of inflammatory cells, immunostaining of various inflammatory cells were performed. As seen in Supplementary Figure S1 online, there were no differences in cutaneous inflammatory infiltrates between young and aged mice. Taken together, these results indicate that otherwise normal-appearing aged skin displays evidence of increased cytokine expression but no inflammatory cell infiltration.

We next determined whether the increased cutaneous cytokine expression in aged skin is accompanied by increased levels of serum cytokines. The serum levels of several cytokines were compared in 12-month-old versus 8-week-old C57BL/6J mice. Compared with young mice, the serum levels of IL-1α, IL-1β, IL-6, and TNF-α were elevated significantly (Figure 2b). Moreover, the levels of serum amyloid A also increased by more than 30% in 12-month-old versus 8-week-old mice (14.5 ± 0.5 μg/ml vs. 19.5 ± 1.9 μg/ml; $P = 0.031$) (Figure 2c).

To determine whether the serum cytokines and amyloid A could originate from the skin, rather than distal organs such as the liver, a known source of serum amyloid A (Upragarin et al., 2005), we next compared mRNA levels of amyloid A and TNF-α in the epidermis and liver of aged mice. Although mRNA levels for both amyloid A and TNF-α increased in aged epidermis, neither of them increased in the liver (Figure 2d), suggesting that the skin could be a major source of serum inflammatory markers, possibly in association with epidermal dysfunction in aged mice. Together, these results indicate that normal-appearing, aged mice exhibit an increase in both cutaneous and serum cytokines, possibly linked to epidermal dysfunction.

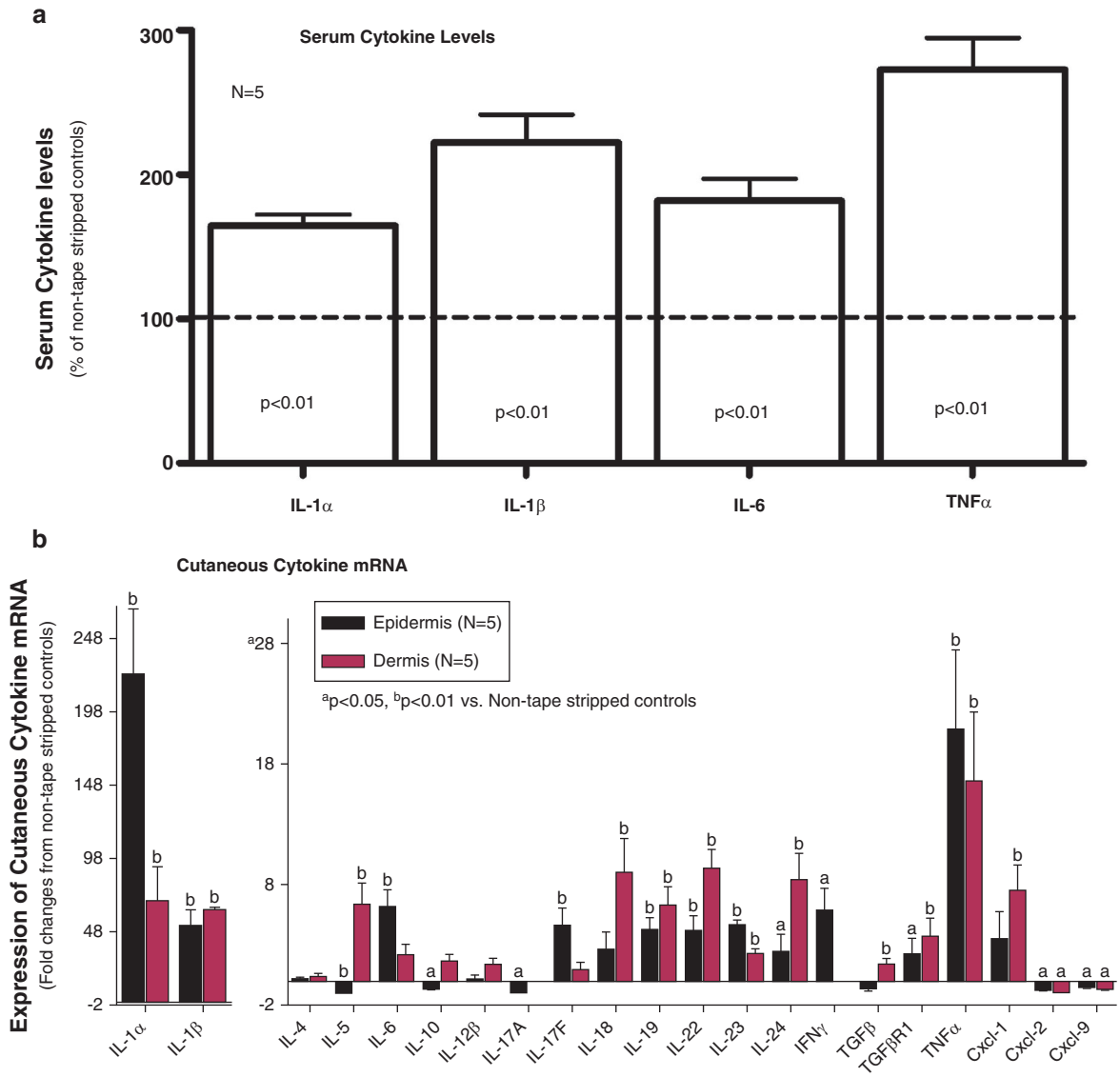

Figure 1. Acute barrier disruption induces not only cutaneous, but also systemic inflammation in normal mice. Both flanks of 8-week-old C57BL/6J mice were repeatedly tape-stripped until transepidermal water loss rates increased 4-fold. One group of non−tape-stripped mice served as normal controls. Three hours after tape-stripping, blood was collected for cytokine analysis (see Methods). Skin samples were collected for determination of cytokine mRNA levels in the dermis and epidermis, separated by brief heating (Feingold et al., 1991). (**a**) Serum cytokine levels, measured by ELISA assay. (**b**) Changes in mRNA levels, measured by quantitative PCR in the dermis and epidermis 3 hours after acute barrier disruption. Data were normalized to non−tape-stripped normal controls, setting normal controls as 100% (dotted line in **b**). A Mann-Whitney two-tailed test was used to determine the significances between tape-stripped versus non−tape-stripped mice. TGF, transforming growth factor; TNF, tumor necrosis factor.

Acute disruption of the epidermal permeability barrier increases both cutaneous and serum inflammatory cytokines in athymic mice

Although our results clearly show that epidermal dysfunction is accompanied by increased serum inflammatory cytokines, the cellular origin of these cytokines is still obscure. To determine whether T cells, one of the major nonepithelial sources of cytokines, contribute to the epidermal dysfunction-induced elevations in cutaneous and serum inflammatory cytokines, we next compared cutaneous cytokine mRNA and serum cytokine levels in normal C57BL/6J versus athymic mice after barrier disruption. As shown in Figure 3a, epidermal mRNA levels of all four cytokines dramatically increased 3 hours after barrier disruption, whereas TNF-α mRNA was undetectable in the dermis of athymic mice, suggesting the importance of T cells in the origin of dermal TNF-α production. Moreover, serum levels of cytokines increased significantly, and to an extent comparable to levels in normal mice, despite T-cell deficiency (Figure 3b and c). These results strongly suggest that T cells are not a major contributor to epidermal dysfunction-induced increases in either cutaneous or serum inflammatory cytokines.

Improvements in epidermal function reduce cutaneous and serum cytokines in aged mice

Our prior studies showed that correction of chronic abnormalities in epidermal permeability barrier function lowers epidermal cytokine levels in murine skin (Wood et al., 1994), suggesting a regulatory role of the epidermal permeability barrier in cutaneous inflammation. Because aged skin

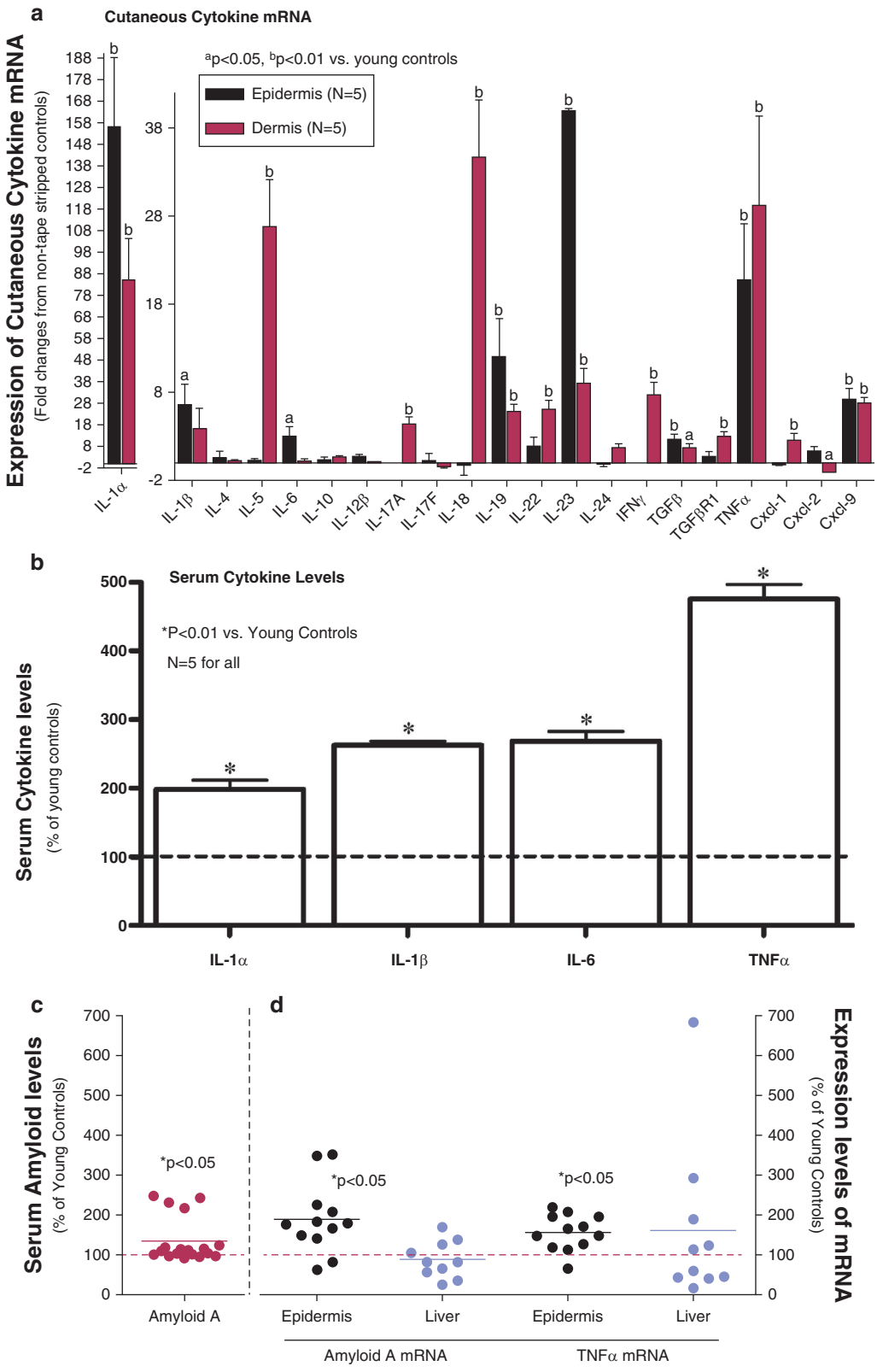

Figure 2. Cutaneous and serum cytokines increase in aged mice. Both flanks of 12-month-old C57BL/6J mice were used in this study. The dermis and epidermis were separated by heat (Feingold et al., 1991). (**a**) Expression levels of cytokine mRNA in the skin. (**b**) Serum cytokine levels. (**c**) Serum amyloid A levels in 7-week-old versus 12-month-old mice. (**d**) mRNA levels for amyloid A and TNF-α in the liver (red dots) and the epidermis (black dots) of 12-month-old mice. Data were normalized to normal young controls, setting normal young controls as 100% (dotted lines). A Mann-Whitney two-tailed test was used to determine the significances between aged and young mice. *P* values were versus the non–tape-stripped normal controls. TGF, transforming growth factor; TNF, tumor necrosis factor.

displays compromised epidermal permeability barrier and reduced stratum corneum hydration (Choi et al., 2007; Ghadially et al., 1995; Man et al., 2009, 2015a), should the increased inflammation in the serum and epidermis of aged mice be primarily due to epidermal dysfunction, correction of the epidermal function should reduce levels of inflammatory markers, not only in the epidermis both also in the serum of aged mice. Therefore, we next determined whether correction of the epidermal functional abnormalities with topical petrolatum lowers cutaneous and serum

cytokine levels in aged mice. We initially treated 12-month-old C57BL/6J mice, which exhibit epidermal permeability barrier dysfunction (Ghadially et al., 1995; Man et al., 2015a), with topical petrolatum, an agent previously shown to improve epidermal permeability barrier function in aged murine skin (Ghadially et al., 1992; Mao-Qiang et al., 1995), three times daily for 10 days. Topical petrolatum treatment lowered mRNA levels of cutaneous cytokines to levels comparable to those that occur in the skin of young mice (Figure 4a, dotted line, setting levels in young mice as 100%). Consistent with these changes in cytokine mRNA, topical petrolatum also induced a 42% reduction in cutaneous TNF-α protein level in aged skin ($P < 0.05$). Finally, serum levels of the same cytokines also significantly declined after topical petrolatum treatments (Figure 4b).

To further confirm the regulatory role of epidermal dysfunction in serum inflammatory cytokines, we next treated the 12-month-old mice topically with glycerol, another agent that improves epidermal permeability barrier function and stratum corneum hydration (Atrux-Tallau et al., 2010; Fluhr et al., 1999, 2003). As shown in Figure 4c, topical glycerol treatments significantly reduced the mRNA levels of several cytokines in aged mouse skin. In parallel, serum levels of the same cytokines also declined significantly (Figure 4d). Collectively, these results show (i) that the increase in proinflammatory cytokines in aged skin and serum can be ascribed to coexistent abnormalities in epidermal function and (ii) that strategies that improve epidermal function in aged skin can attenuate the increases in both serum and cutaneous cytokines.

DISCUSSION

In this study, we found higher levels of proinflammatory cytokines in both the intact skin and serum of aged mice compared with young mice. The increased production of cytokines by aged epidermis likely reflects homeostatic signaling responses to the coexistent permeability barrier abnormality, because (i) an increase in IL-1α levels improves epidermal permeability barrier function by stimulating lipid synthesis, not only in aged skin but also in barrier-compromised young skin (Barland et al., 2004; Jung et al., 2011); (ii) both exogenous IL- 1α and TNF-α accelerate permeability barrier formation during fetal development in vitro (Jiang et al., 2009); and (iii) IL-6 deficiency delays epidermal permeability barrier recovery in mice, whereas exogenous IL-6 improves permeability barrier function in vivo and in vitro (Jiang et al., 2010; Wang et al., 2004). Although permeability barrier disruption increases epidermal cytokine production and release (Tsai et al., 1994; Wood et al., 1992, 1997), correction of the barrier abnormalities by occlusion conversely decreases epidermal cytokine levels (Tsai et al., 1994; Wood et al., 1994, 1997). Thus, increased cytokine production in aged mouse skin, which begins to show functional abnormalities by 12 months of age, likely reflects a compensatory homeostatic response to compromised permeability barrier function. In addition to a permeability barrier defect, aged skin displays low stratum corneum hydration, which likely also contributes to cutaneous inflammation, because reductions in stratum corneum hydration alone increase cutaneous cytokine expression (Denda et al., 1998), whereas, conversely, improvements in stratum corneum hydration relieve cutaneous inflammation (Kikuchi et al., 2003; Xu et al., 2014). Therefore, increased levels of epidermal cytokines likely result from both the compromised permeability barrier function and reduced stratum corneum hydration levels in aged skin.

Although previous studies show that both keratinocytes and fibroblasts can produce proinflammatory cytokines upon stimulation (Ablett et al., 2003; Huleihel et al., 1990, 1993;

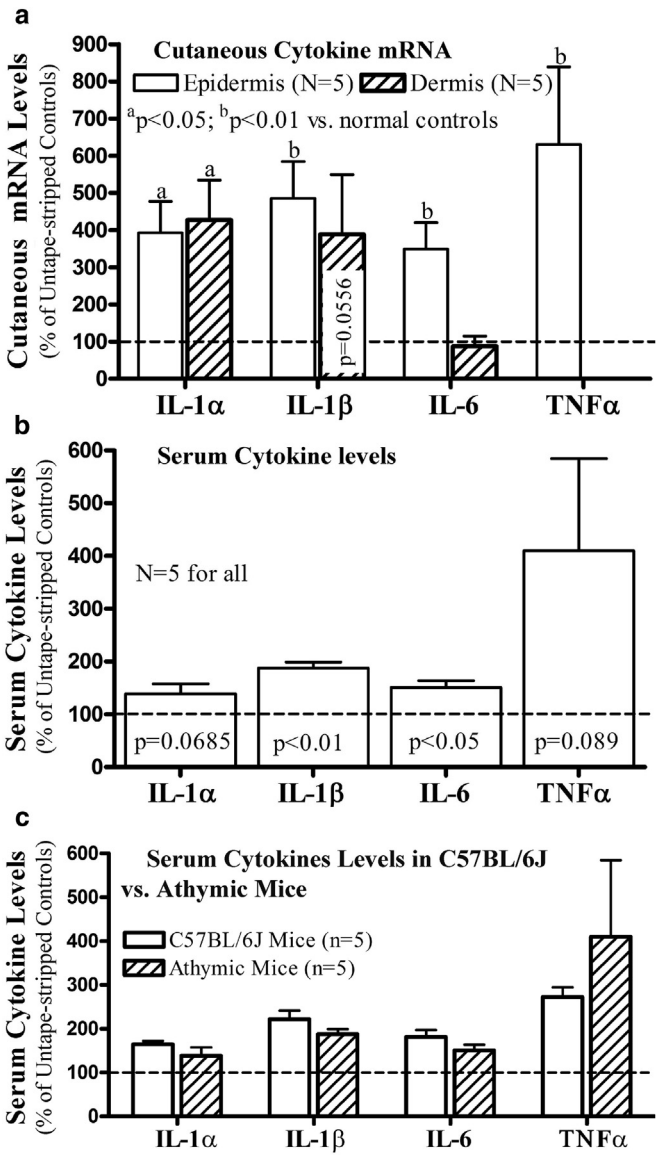

Figure 3. Acute barrier disruption induces increases in cutaneous and systemic cytokines in athymic mice. Both flanks of 8-week-old athymic mice were repeatedly tape-stripped until transepidermal water loss rates increased by 4-fold or greater. Groups of mice and experimental methods were the same as those in C57BL/6J mice. (**a**) Changes in mRNA levels in the dermis and epidermis. (**b**) Serum cytokine levels 3 hours after barrier disruption. (**c**) Changes in serum cytokine levels in athymic versus C57BL/6J mice after acute barrier disruption by sequential tape-stripping. Data were normalized to non–tape-stripped normal controls, setting normal controls as 100% (dotted line). A Mann-Whitney two-tailed test was used to determine the significances between tape-stripped versus non–tape-stripped mice. *P* values were versus the non–tape-stripped normal controls. TNF, tumor necrosis factor.

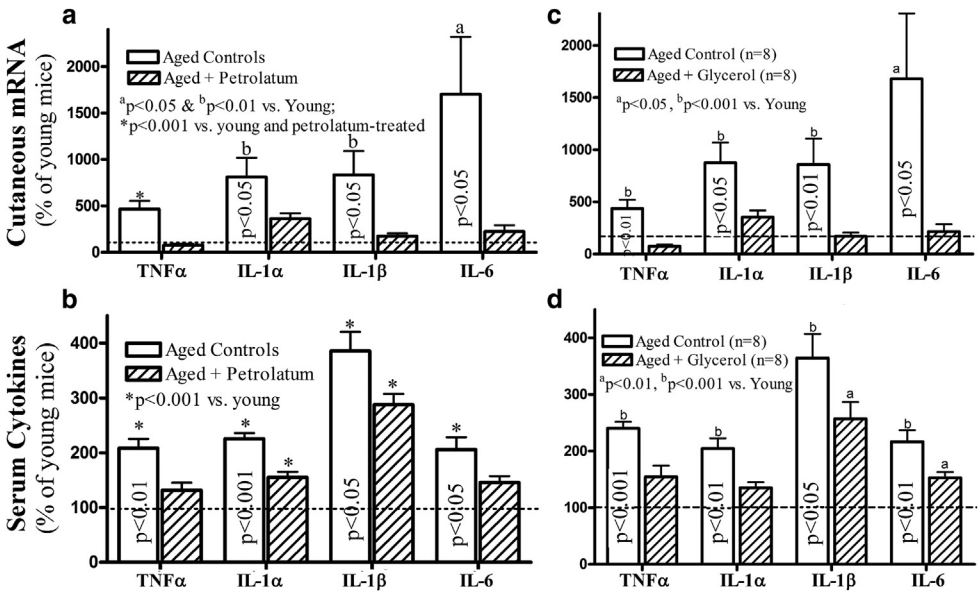

Figure 4. Improvements in epidermal function alleviate not only cutaneous, but also systemic inflammation. 12-month-old C57BL/6J mice were treated topically with either petrolatum or glycerol twice daily for 10 days, followed by collection of blood and skin for analyses of serum cytokines and cutaneous cytokine mRNA. (**a, c**) Expression levels of cutaneous proinflammatory cytokine mRNA levels in aged mice after treatment with petrolatum or glycerol. (**b, d**) Changes in serum cytokine levels in aged mice treated with petrolatum and glycerol, respectively. The data are expressed as percentage of untreated young mice, setting the level of young mice as 100% (dotted line). One-way analysis of was used to determine the statistical significances. The significances are indicated in the figures. N = 8 for all groups. Significances indicated in column bars represent the differences between treated versus untreated aged mice. TNF, tumor necrosis factor.

Köck et al., 1990; Mustafa et al., 2000; Urbanski et al., 1990; Wolf et al., 2013), our results further suggest that the skin is likely an important contributor to the age-associated increase in serum inflammatory cytokines. Although aged mice display elevated serum amyloid A and TNF-α levels, mRNA levels of both amyloid A and TNF-α increase only in the aged skin but not in the liver, a putative source of serum amyloid A (Upragarin et al., 2005). T cells negligibly influence serum inflammatory cytokines after permeability disruption. Because aged skin exhibits reductions in the densities of Langerhans cells, Thy-1$^+$ dendritic cells and eosinophils (Gunin, et al., 2011; Sprecher et al., 1990; Xu et al., 2012), as well as decreased production of TNF-α by macrophages (Agius et al., 2009), the age-associated increase in serum inflammatory cytokines likely instead originates from aged keratinocytes and fibroblasts, both of which are known cytokine producers (Doles et al., 2012; Wolf et al., 2012). However, the increased cytokines could also be ascribed in part to cutaneous mast cells, because aged skin exhibits a greater-than-normal density of mast cells (Gunin, et al., 2011). Nevertheless, further studies will be required to delineate the extent to which different cutaneous cell types contribute to the age-associated increases in serum and cutaneous cytokines.

The prevention and treatment of inflammation-associated systemic disorders in the elderly have been a challenge in part because of their uncertain origins. We show here that aged mice, as in otherwise normal, aged humans (Banerjee et al., 2011; Kim et al., 2011; Mariani et al., 2006), exhibit elevated levels of serum cytokines. Systemic inflammation is a well-known complication of inflammatory skin disorders, such as atopic dermatitis (Yoshizawa et al., 2002) and psoriasis (Jacob et al., 2003; Yamamoto et al., 2013). Pertinently, serum cytokine levels also increase during cold seasons (Valmadrid et al., 2000; Wannamethee et al., 2011), when the epidermal permeability barrier is further stressed by both reduced environmental humidities and low temperatures, which compromise epidermal permeability barrier homeostasis (Denda et al., 1998, 2007; Halkier-Sørensen et al., 1995; Lin, 2009; Muizzuddin et al., 2013). Finally, the link between epidermal dysfunction and serum inflammatory cytokines is most convincingly shown by our observation that correction of epidermal functional abnormality lowers the levels of serum inflammatory cytokines in aged mice. Indeed, we show here that improvements in epidermal permeability barrier function and hydration in aged skin reduce levels of proinflammatory cytokines in both the epidermis and serum. Collectively, the bulk of evidence suggests that permeability barrier dysfunction could contribute to the development of systemic inflammation in aged humans.

Inflammatory skin disorders (e.g., atopic dermatitis and psoriasis) are closely associated with the comorbidity for certain systemic disorders, including cardiovascular disease and diabetes (Donath, 2014; Dregan et al., 2014; Horreau et al., 2013; Marques-Vidal et al., 2013; Silverberg and Greenland, 2015). Acute disruption in epidermal barrier function regulates homeostatic metabolic responses in the underlying epidermis, in part through cytokine and growth factor signaling (Elias et al., 1999). Low stratum corneum hydration, another feature of aged skin, also induces cutaneous inflammation (Denda et al., 1998). However, when these abnormalities persist, epidermal cytokine production eventually produces cutaneous inflammation, which we now show could lead to an increase in serum inflammatory cytokines. It is likely that epidermal dysfunction could play a role, at least in part, in the pathogenesis of chronic systemic diseases, because (i) both cardiovascular disease and diabetes, which affect each other (Muizzuddin et al., 2013;

Valmadrid et al., 2000), occur primarily in aged populations, who inevitably display a defective epidermal permeability barrier (Choi et al., 2007; Ghadially et al., 1995), with higher cytokine levels (Kim et al., 2011; Mariani et al., 2006; Osiecki, 2004) and (ii) defective skin barrier function is associated with type 2 diabetes (Jancin, 2011). However, further clinical studies are required to determine whether enhancement of epidermal functions (permeability barrier and/or stratum corneum hydration) could alleviate/prevent chronic inflammation and certain inflammation-associated disorders in elderly humans.

MATERIALS AND METHODS

Materials

For the studies to determine whether acute barrier disruption influences serum amyloid A and TNF-α levels, and their mRNA in the epidermis and liver, C57BL/6J mice were purchased from Charles River Laboratories (Wilmington, MA) (results in Figures 2c and d). For the rest of studies in this article, animals were from Laboratory Animal Center, Academy of Military Medical Science (Beijing, China) and were housed for 2 weeks in the same room at the animal facility of Tianjin Medical University, China. Cytokine ELISA kits were purchased from R & D Systems (Minneapolis, MN). Mouse amyloid A ELISA kit was from Life Technologies (Grand Island, NY). All primers for cytokines were from Elim Biopharmaceuticals (Hayward, CA). Anti-mouse TNF-α monoclonal antibody was from Santa Cruz Biotechnology (Dallas, TX). Petrolatum jelly (Shanghai Hualing Health Machinery Plant, Xuhui, Shanghai, China) and glycerol (Shanghai Huayin Daily Article Co., Ltd., Minhang, Shanghai, China) were purchased from a local drugstore in Tianjin, China.

Experimental protocols

All animal procedures were approved by the Animal Studies Subcommittee of the San Francisco Veterans Affairs Medical Center and Tianjin Medical University and were performed in accordance with their guidelines. For studies in aged mice, both flanks of 12-month-old C57BL/6J mice were treated topically with 60 μl of glycerol or 200 mg of petrolatum twice daily for 10 days. Because the purpose of using glycerol and petrolatum was to explore the concept that improvement of epidermal function can alleviate cutaneous and systemic inflammation, controls should be treated with agents that affect neither stratum corneum biophysical properties nor epidermal function, including innate immunity. However, all topical agents affect either epidermal function or stratum corneum biophysical property. For example, repeatedly topical applications of water could change stratum corneum hydration, which can affect cytokine expression. Ethanol can alter skin microflora, which could also affect the cutaneous immune system. Therefore, untreated 12-month-old mice and untreated 8-week-old mice served as controls in this study. Eighteen hours after the last topical treatment, both skin and blood samples were collected. The epidermis was separated from dermis by heat separation method as we previously described (Mao-Qiang, et al., 1995). The expression levels of mRNA for proinflammatory cytokine in the epidermis were determined by quantitative PCR. The primer inflammation is detailed in Supplementary Table S1 online. The levels of serum cytokines were measured with ELISA (R & D Systems). In the acute barrier abrogation model, the permeability barrier in both 8-week-old C57BL/6J and athymic mice was disrupted by repeated applications of cellophane tape until a 10-fold increase in transepidermal water loss (Tsai et al., 1994; Wood et al., 1992, 1997). Skin and blood were collected 3 hours after barrier disruption.

Quantitative PCR for mRNA expression

We have shown that the peak increase in epidermal mRNA occurs 3 hours after a single insult to the barrier and returns to basal levels by 24 hours (Wood et al., 1996). We anticipated that the barrier abrogation-induced changes in serum cytokine levels would require a longer time; therefore, we chose both 3- and 6-hour time points in pilot studies. Our results showed that serum cytokine levels had already increased significantly at 3 hours after barrier disruption. Therefore, a 3-hour time point was used in all studies in this article unless otherwise stated. Total cutaneous RNA was isolated from mice as described above using TRI Reagent (Sigma-Aldrich, St. Louis, MO). First, strand cDNA was synthesized from 1 μg of total RNA with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The real-time PCR contained 20 ng of reverse-transcribed total RNA, 450 nmol/L forward and reverse primers, and 10 μl of ×2 LightCycler 480 SYBR Green I Master (Roche Diagnostics, Indianapolis, IN) in a final volume of 20 μl in 96-well plates using Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA). Quantification was performed by the comparative C_T method, with mouse 36B4 used for normalization. Primer sequences are listed in Supplementary Table S1. The relative expression of the mRNAs compared with mRNA in normal young mice was calculated. Data are expressed as percentage of control (setting normal young controls as 100%) (Man et al., 2015a).

Measurements of serum cytokines and amyloid A

The levels of serum cytokines and amyloid A were measured using respective ELISA kits according to the manufacturers' instructions. The relative expressions of cytokines and amyloid A with those in normal young control mice were calculated. Data are expressed as percentage of normal young controls (setting normal young controls as 100%).

Immunohistochemical staining

Cutaneous infiltration of various inflammatory cells was assessed with immunohistochemical staining (antibodies are listed in Supplementary Table S2 online).

Statistics

Data are expressed as the mean ± standard error of the mean. GraphPad Prism 4 software (San Diego, CA) was used for all statistical analyses. Unpaired two-tailed Student *t* test with Mann-Whitney test was used to determine the statistical significances when two groups were compared. One-way analysis of variance with Tukey multiple comparison was used when three groups were compared.

ORCID

Theodora Mauro: http://orcid.org/0000-0003-3623-0070

CONFLICT OF INTEREST

The authors state no conflicts of interest except that invention disclosure has been filed for the concept of preventing/treating systemic disorders using strategies to improve epidermal functions.

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AUTHOR CONTRIBUTIONS

LH, ED, JZ, GM, and DL performed experiments. TMM, GW, KRF, and PME interpreted data and critically reviewed the manuscript. MQM originated concept, designed experiment, analyzed and interpreted data, and wrote manuscript.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2017.01.007.

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