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Chronic Social Stress Ameliorates Psoriasiform Dermatitis Through Upregulation of the Hypothalamic-Pituitary-Adrenal axis

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Abstract

Acute stress is a physiological response of an organism to adverse conditions, contributing to survival; however, persistence through time may lead to disease. Indeed, exacerbation of inflammatory conditions such as psoriasis has been reported to follow stressors in susceptible

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patients. Because chronic stress cannot ethically be elicited in patients under controlled laboratory conditions, we studied genetically modified mice that naturally develop psoriasiform dermatitis, and subjected them to an ethological chronic social contact stress paradigm. Although we found elevated pro-inflammatory neuropeptide production of substance P (SP), calcitonin-gene-related peptide (CGRP) and nerve-growth factor (NGF) mRNA in the Dorsal Root Ganglia (DRG) as well as pro-inflammatory cytokines in response to the social stressor, stress paradoxically prevented the development of the skin lesions. This effect of stress could be reversed by the treatment with glucocorticoid (GC) receptor blockers, suggesting that it was mediated through the upregulation of corticosterone secretion. Extrapolating to humans, the worsening of disease in susceptible patients with psoriasis could be attributed to a defect in the Hypothalamic-Pituitary-Adrenal (HPA) axis with an impaired production of GC during situations of adversity, thus rendering them unable to counteract the pro-inflammatory effects of chronic stressors.

1.0 Introduction

Psoriasis, a chronic papulosquamous skin disease afflicting approximately 3% of the US population (Helmick et al., 2014) is associated with increased levels of distress, depression and anxiety (Rieder and Tausk, 2012). Numerous investigators have found emotional stress an aggravating factor for psoriasis (Seville, 1977, 1978) (Rieder and Tausk, 2012), but precisely defining the role of stressors has proven complex, mostly because studies have been retrospective, based on patient recollection and thus subject to bias (Farber et al., 1986) (Gaston et al., 1991) (Farber and Nall, 1993). Assessment of the effects of *chronic* stressors on disease progression becomes difficult in the absence of controlled laboratory studies, which ethically cannot be performed humans.

Psychological stress occurs when the environmental demands exceed the perception of the ability of a subject to cope with them (Cooper and Quick, 2017). The term stress as originally coined by Selye (Selye, 1936) represented the organism's ability to adapt to acute perceived challenges with a stage of alarm (fight-flight) (Canon, 1939), followed by adaptation, and finally, a stage of exhaustion, disrupting homeostasis and leading to disease (Selye, 1946). McEwen more recently put forth the concept that the organism strives towards a homeostatic balance termed “allostasis”, and the collection of stressors are represented by “allostatic load”, which depending on each individual may lead to adaptation or disease. This framework allows us to understand that stressors may be positive or negative depending on the subject's ability to interpret them and cope, depending on the magnitude of the stressor; the psyche, past history, makeup of the individual and the individual response of the Hypothalamic-Pituitary-Adrenal Axis (HPA). Among the latter, the inability to secrete appropriate levels of cortisol would lead to diseased states (McEwen, 1998).

Animal studies have contributed to elucidating the physiologic immune/endocrine underpinnings of either brief or sustained adverse conditions, showing in general that *acute* stressors (minutes to 2-3 hours) are beneficial to survival, enhancing cutaneous immune functions, redistributing leukocytes to prevent infections and promoting wound healing; while *chronic* stressors have in general deleterious effects on immunity and immune-surveillance (Dhabhar, 2000; Dhabhar, 1997) (reviewed in (Dhabhar, 2013)). For example,

chronic restraint stress facilitates UV-induced skin cancers (Parker et al., 2004), whereas *acute* stress promotes resistance to these same tumors (Saul et al., 2005). Although most studies point towards the immunosuppressive role of chronic stress, the effects are not homogeneous, and the results may be contradictory depending on the measured outcomes, the characteristics of the stressor (physical, psychological, controllable or uncontrollable) and the specific characteristics of the studied individual (species, sex, age, diet, submissive or dominant) (Moynihan, 2003) (Shi et al., 2003). Murine studies have shown contradictory results when evaluating the effects of stress on the development/worsening of atopic dermatitis (AD); some found that as seen in humans (Wright et al., 2005), prolonged water avoidance stress induced the disease in genetically susceptible mice (Amano et al., 2008), and mediated by the upregulation of substance P (SP) stained cutaneous nerve fibers in mice exposed to sound stress (Pavlovic et al., 2008). More recently, Lin et al (Lin et al., 2014) reported improvement of allergic contact and atopic dermatitis in mice exposed to 18 hours of restraint stress. It is important to reiterate that the various animal models of chronic stress (Campos et al., 2013) (Avitsur et al., 2009) may show different and sometimes opposing results depending on the nature of the stressor, particularly when comparing physical (restraint, cold water) with social defeat stress. Commonly applied murine physical stress paradigms, such as prolonged restraint, disrupt the circadian rhythms, feeding, drinking and socialization, leading to a situation that is rarely encountered in the animal's life in the wild. Because stress experienced by humans is mostly of social origin (Stress Statistics, 2017), we chose to examine the effects of a chronic defeat stress model (sensory contact social stress), currently considered the one that most resembles the stressors that lead to human psychopathological disorders (Hammels et al., 2015) (Bartolomucci et al., 2004) on mice genetically modified to express a psoriasiform phenotype. Paradoxically, contrary to the clinical experience of many patients suffering from psoriasis, who worsen under stress, we found that the stressor mitigated the development of the skin disease in these mice.

2.0 Methods

2.1 Husbandry

Male homozygous FVB-Tg(KRT14-Vegfa)3Dtm/J transgenic mice on an FVB/N background were reared in isolator cages and housed in an AAALAC-accredited, climate-controlled facility ($20^{\circ}\text{C} \pm 1^{\circ}\text{C}$ at 50% humidity), with a 12 h light/dark cycle (white lights on from 18:00 to 06:00 h), and fed ad libitum with a commercial diet and water. These mice have been described elsewhere (Poligone, 2012). Briefly the hemizygous FVB-Tg(KRT14-Vegfa)3Dtm/J (Jackson Laboratories, Bar Harbor, ME) were backcrossed to homozygosity of the transgene, establishing a transgenic mouse that develops in 3-6 weeks severe psoriasiform dermatitis in essentially 100% of mice, and thick plaques within a few months, microscopically showing a psoriasiform dermatitis with neutrophilic abscesses (Poligone, 2012). This genetic background has previously been shown to develop an IL-17 dependent psoriasiform dermatitis (Hvid et al., 2008). To differentiate this mouse from the hemizygous mouse, which has a more variable, less penetrant phenotype, established by Detmar et al (Detmar et al., 1998), we refer to it as the Rochester mouse. All studies were approved by the University Committee of Animal Resources of the University of Rochester (UCAR). Animals were housed together, in standard transparent plastic cages four to a cage. They

were randomly assigned to their social conditions, and those in the stress group, moved to the experimental room, which was maintained at a constant temperature of 20° C and a 12-h light:12-h dark cycle (white lights on 20:00–08:00 h local time), and procedures conducted under dim red light.

Eight week old CD-1 mice (Jackson Laboratories, Bar Harbor, ME), allowed to acclimatize for 2 weeks were subsequently housed in individual cages in the experimental room.

2.2 Mouse treatments

2.2.1 Selection and training of aggressive mice—Male CD1 mice were selected and trained by means of a series of alternative confrontations (Vegas et al., 2004). Two interactions were carried out with intruder-submissive mice, in order to enable subjects to acquire an experience of winning, with confrontations being extended up until the observation of the first defense/flee behavior; and three confrontations were carried out with intruder mice, with confrontations being prolonged only until the first attack was observed. Finally, the most homogeneously aggressive subjects were selected, with attack latencies of less than 10 s.

2.2.2 Chronic Sensory Social Contact Stress—Twenty Rochester mice assigned to the stressed group were exposed to the sensory contact social stress model (Kudryavtseva et al., 1991) during a period of 21 consecutive days. Social interaction involved contact with highly aggressive trained and selected CD1 mice (Vegas et al., 2004). During this period of social stress, the experimental subjects were exposed daily to 3 min of agonistic interaction with a different resident dominant CD1 mouse between 8 and 9 AM. The Rochester mice were exposed to a different CD-1 every day for the 21 days. This interchange of pairs prevented mice from becoming habituated to their dominance–submission relationship, and forced them to re-establish it on a daily basis. As a result, the experimental subordinate mice were repeatedly defeated by a different aggressive resident dominant CD1 mouse every day. After each daily confrontation, mice were separated by perforated transparent partitions, located in the same cage in which the confrontation took place (the cage of the dominant CD1 mouse). This permitted the mice to see, hear, and smell each other (sensory contact), but prevented physical contact (outside the 3 min direct confrontation) for a 24 hour period. During the direct interactions, although experimental subjects received some bites, no wounds were evident.

2.2.3 Control intact—This group represents the normal development of the psoriasiform dermatitis in Rochester mice, housed in regular cages at a separate room away from where the stress confrontations took place. These mice did not suffer or witness stress and their cages were not size restricted. This control group was isolated from any known source of stressful stimuli that could activate the physiologic stress response.

2.2.4 Control mice—One 20-mouse control group was housed in similar cages, 2 to a cage, separated by a perforated methacrylate panel in the cages in order to control for the effect of the separator itself and the resulting reduced living environment. Every morning the separator was removed for 3 minutes as with the stress group. These mice were housed in

the same experimental room as the stressed mice. The pairs of mice were siblings, and did not engage in attacks, and did not suffer, but witnessed the ongoing stress, since their cages were in the same room and they could hear and/or smell the ongoing interactions.

2.3 Pharmacologic treatments

Sixty-five Control Intact mice still free of disease were treated with drugs known to ameliorate skin lesions in patients suffering from psoriasis as a further confirmation of the Rochester mouse model. The mice received intramuscular injections of one millilitre of normal saline solution (N=13), dexamethasone (N=16), cyclosporine (N=16) or methotrexate (N=20) three times a week between noon and 3 PM for 3 weeks. The subjects treated with drug interventions were subdivided into groups receiving varying doses of drug in order to generate a dose response curve. The doses that impaired the development of the psoriasiform lesions were 1 to 4 mg for those receiving dexamethasone; 2-10 mg for cyclosporine and 0.6 to 10 mg for those receiving methotrexate. For practical purposes, the results of all effective doses for each drug were shown pooled in Figure 4.

2.4 Blockage of the sympathetic response and glucocorticosteroid receptors

To examine if the mechanism by which the stressor could modulate the skin lesions was due to release of corticosteroids or nor-epinephrine, we performed blocking experiments. Briefly, the β blocker nadolol was dissolved in normal saline to a concentration of 20mg/Kg; 0.1ml was administered subcutaneously once a day for 22 days, starting one day before the commencement of the stress paradigm. The same scheme was used for the administration of the glucocorticosteroid receptor antagonist RU486, which was dissolved in cyclodextrin to a final concentration of 10mg/Kg. A group of 10 mice received the cyclodextrine (vehicle) alone.

2.5 Measure of development of psoriasis phenotype

To assess the development of the skin lesions, we measured the ear thickness, since the dorsal ears are the first sites to manifest the psoriatic phenotype. Photographs of the ears of the psoriatic mice were obtained on day 21 (at the conclusion of the stressor). Ear thickness measures were obtained with a digital spring loaded caliper (Mitutoyo, Aurora, IL) at baseline, on day 21 (at the conclusion of the stressor), and in some animals, 10 days after the end of the stressor (day 31).

2.6 Measure of Corticosterone

We assayed serum glucocorticosteroids as a surrogate measure of stress. Blood was obtained for corticosterone measurements at baseline (prior to the initial stress exposure), following the last confrontation on day 21 and 10 days later (day 31) after the conclusion of stress from 11 Rochester mice and 10 controls. Stressed animals were bled after 30 minutes of stressor exposure, and controls at the same time of the day. Blood samples (50-100 μ l) were collected from the submandibular vein of un-anesthetized animals into heparinized tubes. All samples were collected between 9:00-10:00 AM. Following centrifugation at 4°C for 20 min at 2400 g, plasma was collected and stored at -70°C. Plasma corticosterone concentration (ng/ml) was determined using commercially available ELISA kits (Assay

Designs, Ann Arbor, MI), employing an Opsys MR Microplate Reader (ThermoLabsystems, Chantilly, VA) and Revelation QuickLink Software at 490 nm. Assay sensitivity was 5 pg/tube and intra- and inter-assay variation coefficients were 7 and 8%, respectively.

2.7 Measurement of neuropeptide mRNA in dorsal root ganglia by real time PCR

We isolated DRG's of Stressed and Control mice to examine if stress modulated the expression of sensory cutaneous nerve neuropeptides. Immediately following euthanasia, cervical dorsal root ganglia (DRG) from stressed and unstressed mice were dissected and stored immediately in *RNAlater*® (Ambion, Austin, TX)

Total RNA was extracted using RNeasy® kit (Ambion, Austin, TX) and cDNA was synthesized using SuperScript III First Strand cDNA synthesis kit (Invitrogen, Grand Island, NY). Real-time PCR primers for mouse NFG (Fwd:5'-GCCAAGGACGCAGCTTTCAT-3', Rev:5'-AGTGATCAGAGTGTAGAACAACATGGA-3') mouse Substance P (Tachykinin 1) (Fwd:5'-AAATGTGCGCTATGAGGAATGA-3', Rev:5'-GGAAACATGCTGCTAGGATACAAA-3') mouse Adcyap-1 (mouse-pituitary-adenylate-cyclase-activating-polypeptide-PACAP) (Fwd:5'-GCCCGCTGTCCTACTTAGTC', Rev:5'-AGGTGAACAGGAGACACTGCT-3') mouse CGRP (Fwd:5'-CCAGTGGGTGAGGAGAAAGTC-3', Rev:5'-AAGCAAGACTAGAAGCTCTACTAGG-3') and mouse GAPDH (Fwd:5'-GTCTTCCTGGGCAAGCAGTA-3', Rev:5'-CTGGACAGAAACCCCACTTC-3') were designed using ABI Primer Express Software (Applied Biosystems, Foster City, CA). PCR products were amplified and detected using SYBR Green in an iCycler iQ real-time PCR system (Bio-Rad, Hercules, CA). Relative cDNA amounts were calculated on the basis of the threshold cycle (C_T) value and were normalized to the amount of GAPDH by means of the 2^{-C_T} method (Livak and Schmittgen, 2001).

2.8 Measurement of circulating cytokines

In order to identify specific cytokines involved in systemic inflammatory or immune responses to the stressor, we chose to use an automated platform that would allow the most analytes. Serum cytokine evaluations were performed using custom Millipore Milliplex mouse Cytokine/chemokine kits I, II and III (MCYTOMAG-70K-23, MCYP2MAG-73K-02, MCYP3MAG-74K-05, Millipore, Billerica, MA) in the serum of Control and Stressed mice. The cytokines/chemokines detected for each kit were for kit I (IFN γ , IL-2, IL-4, IL-6, IL-10, IL-13, IL-1 α , IL-1 β , IL-5, IL-7, IL-9, IL-12p40, IL-12p70, IL-15, IL-17, KC, LIF, MCP-1, MIP-1 β , Rantes, TNF- α , VEGF, and MIP-2), for kit II (IL-22, and MIP-3 α), and for kit III (IL-20, IL-23, IL-27, IL-33 and Timp1). Serum specimens were diluted from 1:3 to 1:6 in serum matrix buffer depending on available serum volume and run according to manufacturer's recommendations. In brief, 25 μ L of diluted specimen, serum matrix negative control, or reference standard dilution were mixed with multiplexed bead reagents in a 96 well plate and incubated 16 hours with shaking at 4° C. Plates were then washed twice with kit wash buffer using a BioRad magnetic washer before adding 25 μ L of detection antibody solution. Plates were incubated one hour at room temperature with shaking before adding 25 μ L of streptavidin-Phycoerythrin (PE) and incubating an additional 30 minutes at room temperature with shaking. The plate was then

washed twice with kit wash buffer and 150 μ L of wash buffer was added and mixed prior to reading on a calibrated BioRad BioPlex 200 reader (Luminex® 200™ System). Values were calculated from the standard curve for each multiplexed assay using the BioPlex Manager 5.0 software 5-parameter logistic fit algorithm and reported in pg/mL.

3.0 Results

3.1 Sequential Phases of Study Interventions and Timeline

Figure 1 depicts the timeline of the study. Phase I consisted of a) the assessment of the effect of the stressor on the development of the psoriasisform dermatitis and on serum corticosterone levels, and b) the effectiveness of medicines that improve human psoriasis to have similar effects on the psoriasisform dermatitis in our mouse model of psoriasis. In Phase II we evaluated whether blocking glucocorticoids or adrenergics reversed the effect of the stressor. Finally, in Phase III we evaluated how the administration of the stressor affected circulating cytokines and DRG neuropeptides.

3.2 Phase I

3.2.1 Chronic Sensory Social Contact Stress inhibits the development of the psoriasisform phenotype in Rochester mice—To elucidate the cutaneous effects of chronic stress, we exposed Rochester mice, free of skin lesions, to 21 days of the sensory contact stressor. Prior to commencing the stress, the ear thickness of all mice had a mean of 0.22 ± 0.03 mm ($\pm$ S.D.); during the following 21 days untreated mice are expected to start developing psoriasisform lesions in their ears. We compared the early development of lesions in the different experimental groups (Fig 2). Group A demonstrates the erythematous and swollen ears as expected for Rochester mice that have not been exposed to any intervention, and housed in a room distant from the stressor; these Control Intact subjects had the most ear swelling with a thickness measuring 0.72 ± 0.1 mm (Fig 2 and 3). Less swelling was observed in Group B (Control), which were mice housed in partitioned cages in the same room where the stressor took place, therefore under identical conditions as the stressed mice minus the stress intervention; their ear thickness was 0.64 ± 0.17 mm (Fig 2 and 3). The Stressed mice (Group C) were found to have very little change in their ears compared to the start of the experiment 3 weeks earlier, with a mean thickness of 0.34 ± 0.09 mm (Figures 2 and 3). All measures reflect the mean $\pm$ standard deviation (SD). Although not statistically significant, Control mice had less thickness and erythema than Control Intact mice, which can be attributed to the reduced living quarters, and being housed in the same room as the stressed animals (see Methods section). We included the Control Intact in order to show the psoriasisform dermatitis in normally housed unperturbed Rochester mice.

The stressor significantly impaired the thickness of the ear inflammation ($p < 0.0001$). The mice were observed for 10 days after the end of the stressor, in order to assess if they developed a rebound phenomenon; at day 31 the ear inflammation continued to demonstrate a significant difference ($p < .0001$) between Stressed and Control mice (Figure 3).

3.2.2 The cutaneous effects of Chronic Stress are the result of the activation of the HPA-Axis—We initially examined circulating levels of glucocorticosteroids in order

to determine that indeed the stress protocol induced significant activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis, and that after 21 days of exposure to continuous stress the mice did not experience habituation to the stressor. Raised serum corticosterone (mean $\pm$ SD) was 234 $\pm$ 133 ng/ml as observed in mice 30 minutes following the first confrontation (Day 1) with the CD1 mice (Fig 5). Thirty minutes after the last confrontation at the conclusion of the stress model (Day 21) corticosterone levels remained markedly elevated (mean 275 $\pm$ 159 ng/ml), suggesting that habituation did not occur during this time. Ten days later, stressed mice had serum corticosterone levels that did not differ from the controls. Non-stressed control mice had means of 61 $\pm$ 118 and 27 $\pm$ 30 ng/ml on days 1 and 21 respectively and 12 $\pm$ 12 ng/ml 10 days later. The corticosterone level of the control mice at baseline (6 days prior to commencing the stressor) was elevated, which could reflect the continued adaptation to a new environment. The difference in circulating levels of corticosterone between stressed and unstressed mice during the stress period was highly significant ($p<0.0001$) (Fig. 5). This experiment was repeated with a new set of animals, revealing identical results.

3.2.3 The mouse cutaneous disease improves with drugs used to treat psoriasis—

In order to evaluate if the psoriasiform dermatitis that the Rochester mice developed was susceptible to treatments that are effective in patients with psoriasis, we proceeded to treat 6 week old mice housed in the same conditions as Control Intact described above. Cyclosporine, methotrexate, dexamethasone or saline were administered for 3 weeks; as detailed in Figure 4, all three drug interventions significantly impaired the appearance of psoriasiform dermatitis whereas the saline treated control mice experienced the appearance of the expected inflammation. An example of the ears of a subject treated with 1mg of dexamethasone 3 times a week is shown in Figure 2.

3.3 Phase II

3.3.1 Blocking glucocorticosteroids and catecholamines reverses the effects of the stressor—

In order to address the mechanisms underlying the effects of stress on the rate of development of the psoriasiform phenotype in our mice, we proceeded to examine if blocking the actions of norepinephrine or glucocorticosteroids (GC) could reverse the effects of stress. Groups of mice were injected daily with the β blocker nadolol, the GC receptor blocker RU486 or vehicle. Half of the subjects were exposed to the 21 day contact stress paradigm (Figure 6). Because of the lack of a normal distribution of the ear thickness data, we applied the non-parametric Mann-Whitney U test for the analysis. The overall thickness difference between the non-stressed controls and stressed subjects was significant ($U=144.0$; $p=0.008$; $r=0.39$); no significant differences were observed between stressed animals (vehicle vs. nadolol vs. RU486) nor between control animals (vehicle vs. Nadolol vs. RU486). A significant difference in the thickness between control and stressed animals treated with vehicle (U de Mann-Whitney=15.5; $p=0.013$) and those treated with nadolol ($U=56.0$; $p=0.028$) was observed. While the difference between the former (vehicle treated) presented a large effect ($r=0.48$), the difference between stressed and control nadolol treated animals presented a moderate effect ($r=0.39$). No significant differences were found between stressed and controls that received RU486 ($U=72.5$; $p=0.248$; $r=0.21$), showing that blocking the HPA-Axis reverts the effects of stress on the reduction of the ear thickness, thus

supporting the notion that the outcome of the stressor in preventing the psoriasiform dermatitis is mediated mostly by the secretion of glucocorticosteroids as well as a modest participation of the sympathetic hormones.

3.4 Phase III

3.4.1 Stress enhances the production of cutaneous sensory nerve

neuropeptides—Elevated cutaneous neuropeptides have been reported in psoriatic plaques (Jiang et al., 1998), and prolonged application of capsaicin, which leads to their depletion, improves the overall severity of psoriatic lesions as well as their pruritus (Bernstein et al., 1986; Ellis et al., 1993). Inasmuch as neuropeptides have been suggested to transmit the effect of stressors to the skin (Joachim et al., 2007), we examined if chronic social stress modified the expression of cutaneous neuropeptides in Rochester mice. Because we encountered difficulty in precisely quantifying epidermal nerve fibers stained for peptides in the mice, we resorted to the dissection of cervical DRG to measure the mRNA of PACAP, NGF, SP and CGRP. These peptides are produced in the neuronal cell body and transported antidromically to the skin surface. Compared to the mRNA found in the DRG of control mice, stress induced a significant increase in the production of NGF (1.75 fold), SP (2.36 fold), CGRP (2.34 fold) and moderate of PACAP (1.4 fold) (Figure 7). These results are in accordance with previous observations that exposure of mice to continuous sonic stress for 14 days enhanced between 2-3 fold SP and CGRP fluorochrome stained nerve fibers in the skin and DRG (Joachim et al., 2007; Pavlovic et al., 2008). These results appear to suggest that the lack of inflammation in the stressed mice, despite the increased pro-inflammatory neuropeptides could be due to the suppressive effects of glucocorticosteroids on local immune responses.

3.4.2 Chronic Stress modulates the levels of circulating cytokines—We

measured protein levels of circulating cytokines to evaluate their presence in mice that were starting to express the psoriatic phenotype, as well as to assess if the chronic social stressor was able to modify them. Almost all the tested cytokines were below the detection sensitivity of the assay in the plasma of mice during the baseline period prior to starting the stress, with the exception of KC, MCP-1 and TIMP-1. Non-stressed control mice that were expressing the psoriatic phenotype in their ears at Day 21 did not have any significant cytokine increase compared to the baseline period. This could be expected, since the mice were just starting to develop erythema and cutaneous induration, thus having very mild disease. Therefore, any significant elevation found in the plasma of mice after 21 days of continuous stress can be ascribed solely to the effect of the stressor (Figure 8).

Some cytokines such as IL-6 are known to be up-regulated as a result of social stress (Stark et al., 2002). The 10 samples had a mean of 441 pg/ml with a S.D. of 406, which reflected the wide variation and particularly 2 stressed mice that had undetectable serum IL-6. When comparing the individual subject's circulating IL-6 and corticosterone, there was a positive correlation between these two parameters (Spearman $r=0.7$; $p=0.02$). Similarly, KC, whose human correlate, IL-8 is known to be augmented by chronic stress (Christian et al., 2006), was significantly elevated in stressed mice, and the levels also correlated with corticosterone (Spearman $r=0.68$; $p=0.03$) (Figure 8). MCP-1, which has been previously reported to be a

marker of psychosocial stress in women (Asberg et al., 2009; Lu et al., 2013) was also very elevated in half the stressed mice (900+/-1605 pg/ml). The chronic stress paradigm utilized resulted in significant elevation of circulating IL-22 (Fig 8) and TIMP-1, neither of which had been reported in the past to be associated to activation of the HPA-axis (Figure 8).

4.0 Discussion

The fight-flight response (Canon, 1939), developed through millennia of evolution, activates the HPA-axis and is critical for coping with acute stressors and ensuring survival. However, humans lack physiologic response pathways to deal with the relatively more modern chronic stressors, known to exert significant adverse effects on health and life expectancy (Kiecolt-Glaser and Glaser, 1999; Schulz KH, 2006; Stone, 2000). Prolonged stress triggers enhanced proinflammatory cytokine production and a shift towards Th2-mediated immunity (Elenkov, 2000; Elenkov and Chrousos, 2000; McEwen, 2008; Stark et al., 2001), which leads to worsening of several Th2 associated autoimmune diseases including SLE (Peralta-Ramirez et al., 2004), atopy (Wright et al., 2005) and pemphigus (Cremniter et al., 1998; Morell-Dubois et al., 2008; Ragab et al., 2011). Based on this paradigm, psoriasis, a disease within the Th1/Th17 spectrum (Deng et al., 2016), should improve and not worsen during stress. Furthermore, stressors would also ameliorate the disease by inhibiting the expression of cathelicidin LL-37 (Aberg et al., 2007), believed to play a significant role in the initiation of psoriatic plaques (Lande et al., 2014) (Ritchlin and Krueger, 2016); however, numerous human studies have shown the opposite, that patients with psoriasis deteriorate under stress (Rieder and Tausk, 2012). To address this controversy, we utilized genetically modified mice that spontaneously develop psoriasiform disease, and exposed them to a sensory contact social stress model (Vegas et al., 2004). In contrast to the experience with human patients, we found that 6 week old stressed Rochester mice remained free of lesions after 21 days of continuous stress, whereas non-stressed ones were developing psoriasiform lesions (Fig 2); this suggests that a systemic effect of the chronic social stress ameliorated the development of disease. In order to elucidate potential mechanisms, we pre-treated mice with a glucocorticoid receptor antagonist (RU486) or the β blocker nadolol, and found that administration of RU486 reversed most of the “clinical beneficial” effects of the stressor, albeit a small amount of the effect was seen in those animals pre-treated with nadolol (Fig 6). These findings suggest that the stress-induced corticosterone response (Fig 5) likely accounts for most of the clinical inhibitory effects in the development of the psoriasiform dermatitis observed in mice exposed to chronic social stress. In addition to glucocorticosteroids, stressed animals were noted to have augmented circulating pro-inflammatory cytokines associated to the activation of the HPA-axis such as IL-6, KC and MCP-1. Furthermore, a novel finding of this study was the simultaneous increase of circulating TIMP-1 and IL-22. Interestingly the cytokines that were up-regulated by chronic social stressors are also found to be overexpressed in humans suffering from psoriasis. For example, circulating IL-6 and IL-8 (Zalewska et al., 2006), MCP-1 (Pohl et al., 2009), TIMP-1 (Flisiak et al., 2005) and IL-22 (Michalak-Stoma et al., 2011) are markers of disease activity in patients with psoriasis (Zalewska et al., 2006), decreasing with treatment, and correlating with PASI scores (Elango et al., 2012). It is well known that IL-22 plays a critical role in peripheral inflammation in general, and in particular, the epidermal hyper-

proliferation found in this disease (Wolk et al., 2009). These observations support the notion that chronic stress results in an inflammatory state that, if remained unchecked, should favor worsening of psoriasis.

Studies from various investigators have shown that exposure of humans or rodents to stressors enhances Substance P and CGRP in cutaneous sensory nerve fibers as well as in DRG cells (Harvima et al., 1993; Joachim et al., 2007; Peters et al., 2005), and that these peptides correlate with psoriasis (Saraceno et al., 2006). Based on these observations, we set out to examine the expression of mRNA of various neuropeptides in the Dorsal Root Ganglions (DRG) of Stressed and Control mice. We found significant increases of CGRP, SP and NGF mRNA in DRG of stressed mice compared to non-stressed controls, suggesting a role for these peptides as a link between CNS and the skin, transmitting the effects of stressors to peripheral tissues. For years investigators observed that accidentally sectioning sensory nerves led to local clearance of psoriasis, suggesting that innervation modulates pathophysiology of psoriatic plaques (Farber et al., 1990; Farber et al., 1986; Zhu et al., 2016). These observations may also explain the findings of upregulation of SP and CGRP in psoriatic plaques (reviewed in (Saraceno et al., 2006)). The contribution of the nervous system is further supported by findings that TRPV1/Na_v1.8⁺TH⁺ neurons are required for the activation of the IL-23 driven IL-17/IL-22 pathway, leading to the formation of psoriasiform plaques following imiquimod application (Riol-Blanco et al., 2014). Studies using a mouse model of psoriasis have shown that, similar to what occurs in patients, sectioning sensory neurons resolved the psoriasiform dermatitis, which reverted when CGRP or a SP receptor agonist were injected to the plaques of the mice (Ostrowski et al., 2011). These findings suggest that neuropeptides mediate the neural maintenance of the psoriasiform inflammation and acanthosis. Furthermore, recent studies showed that the action of CGRP on endothelial cells appears to bias immune responses towards the Th17-pole, (Ding et al., 2016); and that CGRP directly upregulates IL-17 production by Th17-cells through increased expression of IL-23 receptors (Mikami et al., 2012). Interestingly, acute and chronic stressors mediate very different, mostly opposing, neuro-immunological events (Dhabhar, 2013, 1997). Indeed, Kleyn et al (Kleyn et al., 2008) reported that a *brief mild stressor* (Trier test) decreased the expression of cutaneous neuropeptides such as CGRP in psoriasis patients. In contrast, chronic stress-mediated upregulation of neuropeptides in the skin should favor the development or worsening of psoriatic lesions as seen in similar studies that showed that stress enhanced the features of rodent atopic dermatitis (AD), which could not be elicited in mice lacking SP receptors (Pavlovic et al., 2008).

Our findings support the notion that an intact HPA-axis appears sufficient to prevent the pro-inflammatory effects of chronic stress. Contextually, these results are directly counter to common clinical observations that the disease severity in psoriasis patients worsens under conditions of significant stress. Psoriasis patients may exhibit an altered HPA-axis, leading to a blunted cortisol response. More than 20 years ago Sternberg reported that Lewis rats were susceptible to experimental inflammatory diseases due to inherited hypothalamic defects that lead to blunted corticosterone output in response to stressors (Sternberg et al., 1989a; Sternberg et al., 1989b). These findings were subsequently translated to the clinical context; patients with rheumatoid arthritis (Dekkers et al., 2001) or atopic dermatitis (Buske-Kirschbaum et al., 1998; Buske-Kirschbaum et al., 1997) were found unable to sustain an

appropriate cortisol secretion when confronted with acute laboratory stressors. Similar findings were seen in patients with psoriasis (Richards et al., 2005) after administration of a brief stressor to two groups of patients: those who believed that the activity of their psoriasis was influenced by emotional stress, and those who saw no correlation; although the baseline cortisol measures were similar, subjects whose disease fluctuated with stress had significantly lower cortisol levels following the stressor. Therefore, psychosocial stress was found to worsen psoriasis only in the subjects who exhibited an impaired HPA axis response (Richards et al., 2005), as suggested by earlier studies (Thaller et al., 1999). This phenomenon of functional hypocortisolism in inflammatory diseases could be due to defects of the HPA-axis, altered glucocorticosteroid availability or glucocorticoid receptor dysfunctions (reviewed in (Silverman and Sternberg, 2012) (Yang et al., 2012)). A number of studies applying similar social stress paradigms have shown, as also seen in humans (Cohen et al., 2012), the presence of glucocorticoid resistance (Fuertig et al., 2016; Sheridan et al., 2000), which implies an altered inhibitory feedback (Bartolomucci et al., 2004) driven by insensitivity to glucocorticoids and a heightened innate inflammatory signaling (Fuertig et al., 2016). Although to our knowledge there are no studies evaluating glucocorticoid resistance (GR) in psoriasis, investigators have reported IL-17-mediated GR in neutrophils and epithelial cells of patients suffering from forms of asthma (Murcia et al., 2016; Zijlstra et al., 2012); furthermore, pro-inflammatory cytokines have been found to be strong inhibitors at every metabolic step of genomic glucocorticoid functioning (Dejager et al., 2014). Albeit that we did not assess the presence of HPA axis dysfunction or glucocorticoid resistance in the present study, the Rochester mice have preserved levels and sensitivity to glucocorticoids, as indicated by the corticosterone response and the effects of glucocorticosteroid receptor blocking in restoration of the disease. These findings may explain the differences seen in their human counterparts with psoriasis (Richards et al., 2005) as well as other inflammatory diseases (Silverman and Sternberg, 2012). It is our contention that patients with psoriasis respond to stress with disease worsening in those subjects that are unable to mount an appropriate HPA-axis response to counteract the deleterious inflammatory effects of the stressor. The presence of an intact HPA-axis with appropriate glucocorticoid response as observed in our novel mouse model of psoriasis appears to be sufficient to inhibit the appearance of lesions during chronic stress.

The future identification of the molecular mechanism responsible for the HPA axis dysfunction in patients suffering from psoriasis may help to identify those subjects who are more susceptible to the deleterious effects of stress. This could allow for rational psychosocial and psychopharmacological approaches that may provide these patients with the appropriate armamentarium to confront daily stressors (Rieder and Tausk, 2012) (Denby et al., 2014) and possibly ameliorate their disease severity.

Limitations

Studies that utilize a murine model of disease such as this one, suffer from the limitation that, even if supported by clinical, histologic and immunocytochemical evidence, it is impossible to state that the mouse skin lesions are identical to the ones suffered by patients with psoriasis. For this reason, we refer to the murine cutaneous disease throughout the manuscript as “psoriasiform lesions”. Another limitation of this study (and any other using

animal models), relates to whether the stress generated in the inbred mice correlates to stress suffered by humans. To this effect, as discussed in detail in the Introduction section, we chose the sensory contact social stress model because, among the many murine models, it mostly resembles the stress situations found in humans and also shares similar immunological findings.

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Abbreviations

AD	atopic dermatitis
CGRP	calcitonin gene-related peptide
DRG	dorsal root ganglion
GR	glucocorticoid receptor resistance
HPA	hypothalamus-pituitary-adrenal
NGF	nerve growth factor
PACAP	Pituitary adenylate cyclase-activating polypeptide

SP substance P

TRPV1 transient receptor potential cation channel subfamily V member 1

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- Mice were genetically altered to develop a psoriasis-like disease by six weeks of age
- Exposure to chronic stress blocked the development of the psoriasiform skin lesions
- Adrenal corticosteroids were responsible for counteracting the effect of the stress
- Stress increased the production of pro-inflammatory neuropeptides in the DRG
- Stress enhanced secretion of serum IL-6, IL-22, KC, TIMP-1 and MCP-1.
- The activated HPA axis counteracts the effects of CGRP, substance P and cytokines

Figure 1. Representation of the sequential phases (Phase I, Phase II and Phase III) of the study and timeline

Figure 2. Examples of mice under the different experimental conditions

Examples of the inflammation and thickness of the ears of 6 week old mice that were: **(A)** *Control Intact*: un-stressed, housed in a separate room from stressed; **(B)** *Control*: un-stressed, but housed in the same room as stressed and in identically partitioned cages, therefore, does not receive the intervention, but does witness the stress in the room; **(C)** *Stressed*: exposed to the sensory contact stressor for 21 days; **(D)** *Control Intact* (unstressed) mouse as in (A), treated with dexamethasone.

Figure 3. Effects of stress on psoriasiform inflammation

Ear thickness as a measure of inflammation. ■ *Stressed* (N=20), □ *Control* (N=20), and ■ *Control Intact* mice (N=5) were treated as described in Figure 2. Baseline is the mean thickness of all mice the day before the stress began. Day 31 represents the thickness of the ears 10 days after the stress ended and mice were returned to grouped housing, four per cage. ***significance ($p < 0.005$). Means $\pm$ SD.

Figure 4. Pharmacologic Treatments

Control Intact mice (un-stressed) were treated with various agents known to improve human psoriasis. The presence of psoriasiform inflammation was documented as millimeters of ear thickness. Treatments consisted of intramuscular dexamethasone (DEXA), cyclosporine (CSA), methotrexate (MTX) or vehicle 3 times a week. All drugs significantly decreased the psoriasiform inflammation * $p < 0.001$. Means $\pm$ SD.

Figure 5. Chronic stress significantly increased the secretion of corticosterone
Control Handled and Stressed mice were housed as described in the Methods section. The latter were exposed to the social stressor for 21 days.

Figure 6. Stress prevents psoriasiform inflammation through the HPA axis and sympathetic system

Ear inflammation in *Control* mice □(N=30) or *Stressed* mice ■ (N=30) that were injected daily with nadolol (β blocker), RU486 (glucocorticoid receptor blocker), or vehicle. Means $\pm$ SD. Baseline represents the average ear thickness at the beginning of the 21 day experiment, included to illustrate that the relevant measures are those beyond the normal thickness of the ears at baseline.

Figure 7. Effects of stress on neuropeptide expression

Measure of mRNA in DRG of stressed mice. The amount of mRNA for each peptide in the DRG of *Control* (un-stressed) mice was normalized to 1. The results are expressed in fold increase of mRNA found in DRG of stressed mice compared to the control mice. N=5, run in quintuplets and repeated 3 times.

Figure 8. Effects of stress on levels of cytokines

Cytokines were measured by Luminex. Bars represent mean+ S.E.M. The graphs for IL-6 and IL-22 represent the Stress Day 21, and do not include measures at baseline or controls since these had undetectable levels. *** $p < 0.005$