Rural participants raised in the presence of farm animals show less immune activation following acute psychosocial stress

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Key words: Trier Social Stress Test (TSST), urban versus rural, inflammation, interleukin (IL)-6, cortisol, old friends

Short Title: URSS: **U**rban vs. **R**ural **S**tress **S**tudy

ABSTRACT

Urbanization is on the rise, although the urban environment is linked to an increased prevalence of both physical and mental disorders. Human and animal studies suggest that an over-reactive immune system not only accompanies stress-associated disorders, but might even be causally involved in their pathogenesis. Here we show in young (mean age, years, (SD): rural, 25.1 (0.78); urban, 24.5 (0.88)) healthy human volunteers that urban upbringing in the absence of pets (n=20), relative to rural upbringing in the presence of farm animals (n=20), was associated with an exaggerated systemic immune activation following psychosocial stress. Questionnaires, plasma cortisol, and salivary alpha-amylase, however, indicated that the experimental protocol was more stressful and anxiogenic for rural participants. In detail, in response to the Trier Social Stress Test (TSST), participants with an urban versus rural upbringing showed a more pronounced increase in the number of peripheral blood mononuclear cells (PBMCs) and plasma interleukin (IL)-6 concentrations. Moreover, *ex vivo* cultured PBMCs from urban versus rural participants secreted more IL-6 in response to the T cell-specific mitogen concanavalin A (ConA). In turn, anti-inflammatory IL-10 secretion was suppressed following TSST in urban versus rural participants, suggesting immunoregulatory deficits in urban participants following social stress. Together, our findings support the hypothesis that urban upbringing in the absence of pets, in contrast to rural upbringing in the presence of farm animals, increases the vulnerability for stress-associated physical and mental disorders by compromising adequate resolution of systemic immune activation following social stress and, in turn, aggravating stress-associated systemic immune activation.

SIGNIFICANCE STATEMENT

Our results show for the first time that a standardised laboratory psychosocial stressor causes a greater inflammatory response in young healthy participants with an urban upbringing, relative to young healthy participants with a rural upbringing. In view of the known links between persistent inflammatory states and psychiatric disturbances and considering that many stress-associated physical and mental disorders are more prevalent in urban versus rural areas, we feel that our findings are of general interest and significance. Moreover, we feel our study is timely, as urbanization and the associated socioeconomic consequences are increasing.

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INTRODUCTION

More than 50% of the world's population currently lives in urban areas, projected to rise to 70% by 2050, with 50% of the urban population living in cities with more than 500,000 residents (1). At the same time psychiatric disorders are more prevalent in urban versus rural areas (2-7). Given that psychosocial stress is a risk factor for many mental disorders (8), an altered neuronal social processing or an elevated acute cortisol stress response provide two possible distinct mechanisms underlying the higher urban prevalence of psychiatric disorders (9-11). However, urban environments are also known for their increased prevalence of chronic inflammatory disorders, including asthma and allergies (3). Moreover, many stress-associated mental disorders are accompanied by an over-reactive immune system and chronic low-grade inflammation (12, 13). Prospective human and mechanistic animal studies strengthen the idea that an exaggerated immune (re)activity plays a role in the development of mental disorders (12-15). For example, individual differences in interleukin (IL)-6 secretion from *ex vivo* stimulated immune cells predict susceptibility versus resilience to a subsequently applied repeated social stressor in mice, while treatment with an anti-IL-6 antibody increases stress resilience (16). Further, it is known that psychosocial stress promotes systemic immune activation and chronic low-grade inflammation (17, 18), and that IL-6 responses to psychosocial stressors, such as the Trier Social Stress Test (TSST) are exaggerated in those with a diagnosis of major depressive disorder (MDD) and increased early life stress (12). Therefore, another possible mechanism predisposing those with an urban upbringing, relative to those with a rural upbringing, to develop inflammatory disorders in general, and mental disorders in which inflammation has been identified as a risk factor in particular, is an exaggerated inflammatory response following psychosocial stress exposure. Increased inflammation in urban environments may be due to impaired immunoregulation, which is thought to be dependent, at least in part, on reduced exposure, especially during early life (19), to microorganisms with

which mammals co-evolved, as has been proposed by the "Biodiversity" hypothesis (20), "Missing Microbes" hypothesis (21), or "Old Friends" hypothesis (5, 22-26), which all have been evoked to explain the epidemic of inflammatory disease in urban environments. Throughout human evolution, the interactions between these ancestral microbiota and the innate immune system promoted immunoregulation, as they were either part of host physiology (human microbiota), were harmless but inevitably contaminating air, food and water (environmental microbiota), or were causing severe tissue damage when attacked by the host immune system (helminthic parasites) (5, 21). However, microbial biodiversity and, thus, overall contact with environmental and commensal microorganisms that were present during mammalian evolution and that play a role in setting up regulatory immune pathways, is progressively diminishing in high-income countries, particularly in urban areas. The latter is due to sanitation, drinking water treatment, excessive use of antibiotics, changes in diet, feeding of formula milk as a replacement for breast milk, increased caesarean section birth rates, as well as increased time spent within the built environment (21, 24, 27, 28). Of particular interest in this context is a recent study showing increased innate immune system activation in Hutterite compared with Amish farm children, and an ameliorating effect of dust extracts from Amish, but not Hutterite, homes on airway hyper-reactivity and eosinophilia in a mouse model of allergic asthma (29). Living on single-family dairy farms with regular contact with farm animals in Amish farm children further goes along with a 4 and 6 times lower asthma and allergic sensitization prevalence, respectively, compared to living on highly industrialized farms with little contact with farm animals in Hutterite farm children (29, 30). Thus, another critical factor contributing to the diminishing contact with "Old Friends" in both urban and rural areas seems to be regular contact with animals. In accordance with this hypothesis, early exposure to both pets and farm animals is able to reduce the risk of childhood asthma and other inflammatory disorders (31, 32). Immigrant studies further suggest that differential contact with "Old

Friends", particularly during early life, accounts for differences in the prevalence of psychiatric disorders in rural versus urban environments (5, 23, 24).

To test whether urban, compared with rural, upbringing is associated with an increased immune response to social stress, we recruited young, physically and emotionally healthy male participants (SupTab 1), raised during the first fifteen years of life either in a city with more than 100,000 residents and in the absence of pets (urban) or on a farm keeping farm animals (rural). Pets were excluded for urban participants as they potently reduce the risk for inflammatory disorders (31), likely by facilitating "Old Friends" contact. Participants were individually exposed to the TSST (33), and, before and after the TSST, heart rate (HR) and blood pressure were assessed, blood was drawn for collection of plasma and viable peripheral blood mononuclear cells (PBMCs) and saliva samples were collected for determination of alpha-amylase (Fig 1). In addition, mental and physical health status, early life and perceived life stress, and subjective strain induced by TSST exposure were assessed using validated questionnaires (Fig 1; SupTab 2).

MATERIAL AND METHODS

Recruiting: This study was approved by the Ethics Committee of Ulm University and is registered at the DRKS (German Clinical Trials Register, ID DRKS00011236). A commuting accident insurance was installed for participating volunteers. Experimenters were covered by the employer's public liability insurance. For recruitment a flyer was designed asking for healthy male participants between 20 and 40 years of age who grew up (until the age of 15) either in a city with more than 100,000 residents and in the absence of pets (urban: n=20) or on a farm keeping farm animals (rural: n=20). Interested participants were then called, and those who turned out to be physically (asked whether they suffer from chronic physical disorders) and emotionally healthy (Structured Clinical Interview for DSM-IV Disorders, SCID-I (telephone screening); Fig 1; SupTab 2), non-smoking, caucasian, non-drug taking (NSAID, cannabis, etc.),

non-excessive exercising (i.e. <4 h per week), non-traumatized (during early life, adolescence and adulthood), non-acutely (within the last 6 months) bereaved or divorced and had a BMI between 20 and 30, were invited to participate in the present study (SupTab 1). For the actual experiment all participants were asked to abstain from caffeine, any kind of drugs (e.g. analgesics, sleep-inducing drugs, dietary supplements), exercise, alcohol and nicotine for a minimum of 3 days. Furthermore, participants were told to sleep at least 8 h during the night before the experiment and to drink at least 1 l of water on the experimental day itself. In cases of unforeseen illness, test persons were told to delay the experiment. Data were collected between October 2016 and April 2017.

Experimental procedure (Fig. 1): On the test day itself, participants were told to arrive at the laboratory at 1 p.m. and immediately afterwards their current health status was determined and sociodemographic features were assessed (SubTab 1). Only if no signs of illness were reported, the venous catheter (non-dominant arm), as well as the blood pressure and heart rate monitor (dominant arm) were placed (-60 min time point) in a room adjacent to the TSST room. Immediately prior to catheterization, participants had been informed about possible side effects of the catheterization and TSST procedure by the PIs. Afterwards, basal physical and emotional health statuses of the participants were assessed, employing validated questionnaires (List of complaints for quantitative analysis of current bodily and general complaints (BL); State-(Trait-)Anxiety-Inventory (STAI-S) Questionnaire. Before (-5 min) and after (5, 15, 60, 90 and 120 min) the TSST, heart rate and diastolic (D) and systolic (S) blood pressure (BP) were assessed (for calculation of median arterial pressure according to the formula: DBP + (SBP-DBP)/3), blood was drawn in ethylenediaminetetraacetic acid- (EDTA) and lithium heparin-coated monovettes for collection of plasma and peripheral blood mononuclear cells (PBMCs), respectively, and saliva samples were collected for determination of alpha amylase concentration. After the 5^{th} blood draw (90-min time point), STAI-S was used again to assess

subjective strain induced by the TSST procedure. After the 6th blood draw (120 min) the catheter was removed and mental health status (Hospital Anxiety and Depression Scale - German Version, HADS-D; SCID-I (affective part)), early life (Childhood Experience of Care and Abuse Questionnaire, CECA-Q; Childhood Trauma Questionnaire, CTQ) and perceived life stress (Perceived Stress Scale-4, PSS-4) were assessed using validated questionnaires. Of note, although PBMC *ex vivo* culturing and plasma sampling were done and samples stored (-80 °C) at each time point (-5, 5, 15, 60, 90 and 120 min), plasma cytokine concentrations were measured only at -5, 60, 90, and 120 min and cytokine concentrations in the supernatants only at -5 and 120 min.

TSST: Acute psychosocial stress was induced using the TSST, which was performed as described earlier (33), with minor modifications. Briefly, the test consisted of a 3-min preparation phase for a simulated job interview, followed by a 2-min completion of the Primary Appraisal Secondary Appraisal Scale (PASA; ~ 2 min), a 5-min public speaking task, and a 5-min arithmetic task. For further details, see SupSec 1.1.

Blood pressure and heart rate: Blood pressure and heart rate of the participants were determined at time points -5, 5, 15, 60, 90 and 120 min, using a digital brachial blood pressure monitor (Boso Medicus Control, Bosch + Sohn GmbH und Co. KG, Jungingen, Germany). For further details, see SupSec 1.2.

Blood draw: Blood (7.5 ml at each time point) was collected from an indwelling venous catheter in the non-dominant arm (inserted at -60 min) at time points -5 min (5 min before the start of the TSST), 5 min (5 min after termination of the TSST), 15 min, 60 min, 90 min and 120 min into chilled EDTA-coated monovettes. The latter were centrifuged (1000g/ 15 min, 4 °C) immediately after each blood draw and plasma was aliquoted and stored at -80 °C until

189 further processing. Additionally, 9 ml of blood were collected at each time point into lithium-
190 heparin-coated monovettes and stored on ice until blood from all time points was drawn for
191 subsequent isolation and *ex vivo* stimulation of PBMCs.
192
193 **PBMC isolation and stimulation:** Nine ml blood were transferred from lithium-heparin-
194 coated monovettes into Leucosep™ tubes (Greiner Bio-One GmbH, Frickenhausen, Germany),
195 which were prepared with Ficoll® Paque (GE Healthcare Life Sciences, Freiburg, Germany)
196 according to the manufacturer's instructions beforehand. The number of viable PBMCs
197 (identified using trypan blue staining) was determined using an automated cell counter (TC20™
198 Automated Cell Counter, BIO-RAD Laboratories, Munich, Germany). 2.5 x 10^5 cells were then
199 cultured in 96-well plates, either under basal conditions or in the presence of concanavalin A
200 (ConA; final concentration: 2.5 µg/ml) or lipopolysaccharide (LPS; final concentration: 1
201 µg/ml) at 37 °C and 5% CO_2 for 24 hours. Supernatants were collected afterwards and stored at
202 -80 °C until further analysis. For further details, see SupSec 1.3.
203
204 **Enzyme-linked immunosorbent assay (ELISA):** Plasma samples and supernatants from
205 PBMC stimulations were analyzed using commercially available ELISA kits according to the
206 manufacturers' instructions. For further details, see SupSec 1.4.
207
208 **Determination of salivary alpha-amylase concentrations:** Salivary alpha-amylase as a
209 surrogate marker of sympathetic nervous system activity was measured as described earlier (34).
210 For further details, see SupSec 1.5.
211
212 **Statistics:** For statistical comparisons, the software package IBM SPSS statistics (version 22.0)
213 and Stata version 14.2 SE (StataCorp. 2016, College Station, TX: StataCorp LP) were used.
214 Extreme outliers were identified using Grubbs' test and excluded from further analysis (PBMC

counts: *n*=1 (urban); plasma IL-6: *n*=2 (rural), *n*=1 (urban); *ex vivo* PBMC stimulation: IL-6 basal, *n*=1 (rural), *n*=1 (urban); IL-6 ConA, *n*=2 (rural), *n*=2 (urban); IL-10 LPS, *n*=1 (rural); plasma cortisol: *n*=3 (rural), *n*=2 (urban); alpha amylase: *n*=1 (rural), *n*=1 (urban); mean arterial pressure: *n*=1 (rural); heartrate: *n*=1 (urban). Data sets were subsequently analyzed using chi^2 test (nominal scaled data), parametric Student's *t*-test (one factor, two independent samples) or a linear mixed model approach. A linear mixed model analysis was used because it has several advantages over the repeated measures ANOVA when analyzing repeated measures data, including (1) the accommodation of multiple missing data values, (2) the ability to more effectively estimate model parameters in unbalanced experimental designs, (3) more flexibility in model fitting through the objective selection of covariance structures that better fit the correlations between data points, and (4) the ability to model nonlinear changes in a dependent variable across time and treatment (35-38). The latter was followed, when a significant main effect for one factor or an interaction between the two factors was found, by post hoc analysis using Bonferroni pairwise comparison. Data are presented as mean + or ± SEM. The level of significance was set at $P \leq 0.05$.

RESULTS

Sample characteristics

Experimental groups did not differ in any of the socioeconomic parameters assessed, but significantly more rural versus urban participants had regular contact with pets and/or farm animals during adulthood (SupTab 1). Furthermore, we did not detect any differences in early life (CECA-Q, CTQ) and perceived life stress (PSS-4) between urban and rural participants (Fig 1; SupTab 2). SCID-I and BL scores (Fig 1; SupTab 2), assessed during telephone screening (SCID-I), at -60 min prior to TSST (BL), or at 120 min after TSST (SCID-I), indicated that mental and physical health status were also not affected by upbringing.

Effects of upbringing and/or TSST on emotionality

Anxiety levels in the STAI-S (State-(Trait-)Anxiety-Inventory), both at -60 ($P = 0.014$) and 90 ($P = 0.005$) min, and in the HADS-D (Hospital Anxiety and Depression Scale-German Version, $P = 0.027$) were increased in rural versus urban participants, as well as scores in threat ($P = 0.005$), challenge ($P = 0.032$), primary appraisal ($P = 0.004$) and stress-index ($P = 0.025$) in the PASA (Primary Appraisal Secondary Appraisal Scale) (Fig 1; SupTab 2).

Effects of upbringing and/or TSST on systemic immune activation

Basal numbers of viable PBMCs were not different in participants with an urban or rural upbringing. However, compared to basal values (at -5 min), while PBMC counts in rural participants were increased only transiently at 5 min ($P = 0.015$), PBMC counts in urban participants were increased at 5 ($P < 0.001$), 60 ($P = 0.023$), 90 ($P = 0.018$) and 120 min ($P < 0.001$; factor time: $F_{5, 185} = 12.621$, $P < 0.001$), resulting in higher PBMC counts in participants with an urban, versus rural, upbringing at 5 ($P = 0.015$), 60 ($P = 0.040$) and 120 min ($P = 0.023$; factor upbringing: $F_{1, 37} = 5.272$, $P = 0.027$; factor time x upbringing: $F_{5, 185} = 2.112$, $P = 0.066$; Fig. 2A). Basal plasma IL-6 concentrations were not different in participants with an urban or rural upbringing. Plasma IL-6 concentrations in both urban and rural subjects were increased compared with respective basal values (factor time: $F_{3, 105} = 23.836$, $P < 0.001$; Fig. 2B) at 60 (urban: $P < 0.001$; rural: $P < 0.001$) and 90 min (urban: $P < 0.001$; rural: $P < 0.001$). However, participants with an urban upbringing, relative to those with a rural upbringing, showed a prolonged increase in plasma IL-6 concentrations compared to respective basal values at 120 min ($P < 0.001$), consistent with an overall interaction between upbringing and time (factor time x upbringing: $F_{3, 105} = 3.118$, $P = 0.029$; Fig. 2B).

Effects of upbringing and/or TSST on *ex vivo* PBMC cytokine release

Basal *ex vivo* IL-6 secretion from isolated PBMCs (Fig. 3A) was comparable between participants with urban and rural upbringing, and unaffected by TSST exposure. *Ex vivo* IL-6 secretion from isolated PBMCs during ConA (factor upbringing: $F_{1, 37} = 13.728$, $P = 0.001$; Fig. 3C), but not LPS (Fig. 3B), stimulation was significantly increased in participants with an urban upbringing versus participants with a rural upbringing at -5 ($P = 0.012$) and 120 min ($P = 0.029$). *Ex vivo* IL-10 secretion from isolated PBMCs was lower following TSST exposure, but only in participants with an urban upbringing, both in the presence of LPS (factor time x treatment: $F_{1, 37} = 7.922$, $P = 0.008$; $P = 0.035$; Fig 3D) and ConA (factor time: $F_{1, 38} = 12.399$, $P = 0.001$; factor time x treatment: $F_{1, 38} = 4.518$, $P = 0.040$; $P < 0.001$; Fig 3E).

Effects of upbringing and/or TSST exposure on hypothalamic-pituitary-adrenal (HPA) axis, sympathetic nervous system, and cardiovascular system

Rural versus urban upbringing was associated with higher absolute plasma cortisol concentrations both at basal (-5 min; $P = 0.039$) and at the 5 min ($P = 0.030$) time point (factor upbringing: $F_{1, 33} = 5.246$, $P = 0.029$; Fig. 3A). Compared with basal values, plasma cortisol concentrations were increased in both urban and rural participants (both $P < 0.001$) at 5 min (factor time: $F_{5, 165} = 26.978$, $P < 0.001$; Fig 4A), with a comparable delta increase (5 min – basal) between the groups (Fig 4A inlay). Basal (-5 min) salivary alpha amylase concentrations were not different in participants with an urban or rural upbringing. Salivary alpha amylase (factor time: $F_{5, 180} = 25.723$, $P < 0.001$; Fig. 4B) was increased in both groups at 5 min (urban: $P < 0.001$; rural: $P < 0.001$) and/or 15 min (rural: $P = 0.001$) compared to respective basal values. Basal mean arterial blood pressure was not different in participants with an urban or rural upbringing. Mean arterial blood pressure (factor time: $F_{5, 185} = 59.241$, $P < 0.001$; Fig. 4C) was increased in both groups at 5 min (urban: $P < 0.001$; rural: $P < 0.001$) and 15 min (urban: $P = 0.001$; rural: $P < 0.001$) compared to respective basal values and a main effect of time was found for heart rate ($F_{5, 185} = 14.810$, $P < 0.001$; Fig. 4D).

DISCUSSION

Here we showed an increased systemic immune activation in response to a standardized laboratory social stressor in healthy participants with an urban upbringing in the absence of pets, relative to healthy participants with a rural upbringing in the presence of farm animals, even though questionnaires, plasma cortisol, and salivary alpha-amylase indicated that the experimental protocol was more stressful and anxiogenic for rural participants. These data are in line with the "Biodiversity", "Missing Microbes", and "Old Friends" hypotheses, which propose that the rapid rise in inflammatory physical and mental diseases in modern, urban societies is due in part to a lack of exposure to immunoregulatory microorganisms in urban environments (5, 21, 23-26). Another possible explanation for the increased immune reactivity following TSST in participants with an urban upbringing, relative to participants with a rural upbringing, might be that natural landscapes provide a stronger positive health effect compared to urban landscapes, resulting in accelerated short-term recovery from stress or mental fatigue, faster physical recovery from illness and long-term overall improvement on people's health and well-being (39). A brief nature experience, a 90-min walk in a natural, but not urban, setting, further decreases both self-reported rumination and neural activity in the subgenual prefrontal cortex (sgPFC) (40), a brain region previously shown to respond with decreased activity to Montreal Imaging Stress Task exposure in rural versus urban participants (9).

Acute psychosocial stress was induced using the TSST (33), well-known for its ability to elevate PBMC counts (41) and plasma concentrations of IL-6 (12). Consistent with these earlier studies, TSST exposure in the current study increased the number of viable PBMCs in both rural and urban participants 5 min following stressor termination, when compared to respective basal values (at -5 min). However, while PBMC counts in rural participants were increased only at 5 min, PBMC counts in urban participants were increased at 5, 60, 90 and 120 min following

TSST, indicating a more pronounced immune activation in urban versus rural participants in response to a social stressor. This is further supported by the fact that PBMC counts of urban participants were elevated compared with rural participants at the 5, 60 and 120 min time points following TSST exposure. Although we did not perform differential blood counts in the current study, increased lymphocyte and unaffected monocyte counts following TSST have been reported in previous studies (42, 43) suggesting that the exaggerated PBMC mobilization in urban versus rural participants in the present study is mainly mediated by the lymphocyte compartment. Again, consistent with well-known TSST effects (44-46), plasma IL-6 concentrations were increased in both urban and rural participants 60 and 90 min following TSST compared with respective basal values. Importantly and consistent with the exaggerated stress-induced PBMC mobilization, this increase was again more pronounced in urban versus rural participants. While plasma IL-6 concentrations in rural participants peaked at 90 min and were not different from baseline at 120 min, levels in urban participants were elevated until at least 120 min, indicating a prolonged inflammatory response following TSST exposure. Changes to plasma IL-6 after 120 min or before 60 min were not expected (44) and, thus, not studied here, but our results suggest that increased IL-6 might persist beyond the 120 min time point in urban participants. As plasma IL-6 at baseline, 60 and 90 min did not differ between the groups, basal and acute stress-induced immune activation seem to be unaffected by upbringing, whereas immunoregulatory capacity responsible for adequate resolution of stress-induced immune activation seems to be compromised in urban participants.

Basal *ex vivo* IL-6 secretion between PBMCs from participants with urban and rural upbringing was comparable and TSST independent, suggesting that urban versus rural effects on plasma IL-6 concentrations were due to changes in circulating PBMC numbers rather than to individual cell activity. In contrast, *ex vivo* IL-6 secretion during ConA, but not LPS, stimulation was significantly increased in urban versus rural participants at baseline conditions and 120 min following TSST. The latter suggests increased cellular reactivity of the adaptive (ConA), but

not the innate (LPS), immune system towards immunologic stimuli in urban versus participants, which is in line with the fact that, based on previous studies (42, 43), mainly lymphocytes are mobilized by TSST. Although TSST exposure in the present study did not sensitize proinflammatory *ex vivo* cytokine secretion as described earlier (41), increased *ex vivo* cytokine secretion towards immunologic stimuli has been reported for both depressed (47) and posttraumatic stress disorder (PTSD) patients (13). Of note, as these studies employed immunologic stimuli specific for either T cells and, thus, adaptive immunity (i.e. phytohaemagglutinin) (47), or for monocytes and, thus, innate immunity (i.e. LPS) (13, 48), it remains to be investigated whether different psychiatric disorders like anxiety disorders, mood disorders, as well as trauma- and stress-related disorders go along with, or are promoted by, activation of either the innate or adaptive immune system. Data from studies on healthy school teachers indicate that not only lymphocytes but also monocytes of individuals that experience high levels of effort-reward imbalance are more likely to show a pronounced inflammatory response to a mitogen signal (48, 49).

Interestingly, in support of the above reported plasma IL-6 findings suggesting immunoregulatory deficits in urban participants, *ex vivo* PBMC IL-10 secretion was inhibited by TSST only in urban participants, both in the presence of LPS (Fig 3D) and ConA (Fig 3E). As the stress protective and immunoregulatory effects of repeated immunization with *Mycobacterium vaccae*, a soil-derived, saprophytic bacterium with immunoregulatory and anti-inflammatory activity, are mediated by the induction of Treg and IL-10 secretion (50), and as IL-10 deficient mice are prone to develop inflammatory disorders (51), these findings are in accordance with the increased risk for both inflammatory somatic and mental disorders in urban versus rural participants (2, 3). Importantly, increased inflammatory TSST responses have been also reported in other young healthy individuals at risk for mental disorders, with response magnitudes predicting disease incidence (45, 52-55).

In contrast to the immunologic results reported above and in contrast to findings reported by Steinheuser et al. (10), the TSST reactivity of the HPA axis and the sympathetic nervous system and cardiovascular system were comparable, or significantly more pronounced, in rural versus urban participants. In detail, compared to respective baseline values, plasma cortisol concentrations were increased in both urban and rural participants 5 min following TSST, with a comparable delta increase between the groups. In line with these findings, salivary alpha amylase, a surrogate marker for sympathetic nervous system activity (34), and medial arterial pressure were increased in both groups at 5 and/or 15 min, compared to respective basal values, and a main effect of time was found for heart rate. Interestingly, rural versus urban upbringing was associated with higher plasma cortisol concentrations, both at baseline and 5 min after the TSST; likewise, rural versus urban upbringing was associated with a delayed normalisation of salivary alpha amylase, suggesting that the experimental setup and procedure was more stressful and aversive for participants with a rural upbringing compared with participants with an urban upbringing. This hypothesis is supported by increased anxiety levels in the STAI-S, both at -60 and 90 min, and in the HADS-D, as well as with increased scores in threat, challenge, primary appraisal and stress-index in the PASA reported by rural versus urban participants. Given that glucocorticoids during acute stress have been shown to facilitate immune activation (56-60), it is unlikely that the transiently elevated basal cortisol concentrations in rural versus urban participants are involved in mediating the decreased TSST-induced immune activation in rural compared with urban participants. This is further in line with data showing that stress-induced mobilization of bone marrow myeloid cells is mediated by ß3-adrenergic signaling (61) and data suggesting that TSST-induced nuclear factor kappa B (NF-κB) activation in PBMCs is likely mediated by adrenoceptor-mediated signaling (62). However, TSST-induced nuclear factor kappa B (NF-κB) activation in PBMCs was also shown to negatively correlate with plasma cortisol response (63). However, as the significant main effect for factor upbringing on plasma cortisol levels did not hold when controlling for BMI, education, income, and current

daily contact with pets and/or farm animals, the effects of upbringing on plasma cortisol levels were dependent on one or more of these covariates.

Thus, although an increased HPA axis (re)activity has been associated with several psychiatric disorders (64), our data do not support, or even are contrasting to, the hypothesis that the increased prevalence of mental disorders in urban versus rural areas (2, 5-7) is due to an exaggerated HPA axis (re)activity. These data are consistent with recent findings that endocrine and autonomic stress systems do not impact the emotional stress experience after psychosocial stress (11). Furthermore, we did not detect any differences in early life (CECA-Q; CTQ) and perceived life stress (PSS-4) between urban and rural participants, making it also unlikely that "a more demanding and stressful social urban environment" (9) contributes to or even mediates the increased disease prevalence in the urban versus rural population in other studies (2, 5-7). Of note, SCID-I and BL scores, assessed during telephone screening (SCID-I), at -60 min prior to TSST (BL), or at 120 min after TSST (SCID-I), indicated that mental and physical health status were not affected by upbringing.

Interestingly, highly industrialized farming with low contact with farm animals, relative to traditional farming with regular contact with farm animals, is paralleled by increased innate immune system activation and higher prevalences of asthma and allergic sensitization, conditions that are characterized by dysregulation of both innate and acquired immune function (29, 30). Thus, it is likely that the protective effect of rural versus urban upbringing on TSST-induced immune activation seen in the present study is rather due to differences in regular animal contact during early life than to the degree of urbanization per se between the groups. The latter interpretation would be in line with data indicating that incidence rates of certain cancer types as well as cardiovascular disorders in the US, both representing illnesses associated with inflammation, are higher or at least decreasing more slowly in rural compared with urban environments (65, 66). The trend towards more and more industrialized farming and mechanization of farm work in the last decades (67, 68) and, consequently, the lack of regular

and intense animal contact, might explain why many earlier studies showed lower incidence rates of these disorders in rural versus urban areas prior to 2007 (66).

Our study has several strengths but also some limitations that warrant consideration. Notable strengths are the use of an objective and highly standardized stress test, the combination of both *in vivo* and *ex vivo* techniques to assess immune (re)activity, and repeated *in vivo* measures of physiological and immunological parameters in the same individuals over a period of 120 min, taking into account the temporal dynamics of the stress response. Another strength is that all significant main and interaction effects reported in the current manuscript, except the main effect for factor upbringing reported for plasma cortisol levels, were still detectable after adding *BMI, High income, High education and current daily contact with pets and/or farm animals* as covariates (linear mixed model approach). This strongly argues for the robustness of our findings and for the critical role of an urban versus rural upbringing in the absence of pets or the presence of farm animals, respectively, on these parameters. One limitation of the study is the cross-sectional design of our study and the relatively small sample size, which, nevertheless, is representative of the sample sizes in the few previous studies available assessing plasma cytokine levels following TSST exposure (12, 45, 49, 69). Another limitation of the study is that only male participants were used. Given that women are more likely to develop mood disorders compared with men (70), future studies need to address sex differences in rural versus urban effects on TSST-induced immune responses. Additional limitations are that we did not take into account possible differences in participants' mode of delivery at birth, antibiotic usage during first years of life, feeding of formula milk as a replacement for breast milk, or diet. All these factors are well-known to affect the microbiome and microbiome-gut-brain axis, and consequently immune (re)activity, stress responsiveness and behavior (19, 71-74). Given the pronounced differences in TSST-induced PBMC mobilization between urban and rural participants, another limitation of the current study is that we do not have cell compositional data that would allow us to draw conclusions on the particular cell type(s) mediating this effect.

Despite these limitations, we believe that our experimental approach contributes significantly to our understanding of possible biological mechanisms underlying increased risk for inflammatory diseases, as well as increased vulnerability to mental health where inappropriate inflammation is thought to be a risk factor, for those raised in urban areas. Our findings reveal for the first time an increased systemic immune activation and a compromised resolution of inflammation in urban versus rural participants when exposed to an acute social stressor, likely mediated by differences in early animal contact, although validated questionnaires and plasma cortisol data clearly argue for rural participants perceiving the experimental procedure as more stressful and anxiogenic.

ACKNOWLEDGEMENTS

The authors gratefully thank P. Hornischer and U. Binder (both from the Laboratory for Molecular Psychosomatics, Clinic for Psychosomatic Medicine and Psychotherapy, University Ulm, 89081 Ulm, Germany) and Dr. M.T. Rojewski (Institute for Transfusion Medicine, University Ulm, 89081 Ulm, Germany) for their excellent technical assistance. The authors further are grateful to L. Hackl, B. Schembera, P. Marquardt, A. Bauer, B. Häringer, T. Richter, L. Hermann, M. Tisch, M. Zeh, F. Weinreich, H. Holzrichter and J. Kunze (all University Ulm, 89081 Ulm, Germany) for participating as jury members in the TSST and to M.C. Flux (Department of Integrative Physiology and Center for Neuroscience, University of Colorado Boulder, Boulder, CO 80309, USA) for assistance with statistical analysis. Furthermore, the authors would like to thank Prof. Dr. M. Wirsching (University of Freiburg) for his critical and helpful project discussions.

FINANCIAL DISCLOSURES

All authors have nothing to declare and no conflicts of interest.

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FIGURES & FIGURE LEGENDS

Fig 1. Diagrammatic illustration of the experimental procedure. Abbreviations: BL: List of complaints for quantitative analysis of current bodily and general complaints; CECA-Q: Childhood Experience of Care and Abuse Questionnaire; CTQ: Childhood Trauma Questionnaire; HADS-D: Hospital Anxiety and Depression Scale-German Version; PASA: Primary Appraisal Secondary Appraisal Scale; PBMC: peripheral blood mononuclear cell; PSS-4: Perceived Stress Scale-4; SCID-I: Structured Clinical Interview for DSM-IV Axis I Disorders; STAI-S: State-(Trait-) Anxiety-Inventory; TSST: Trier Social Stress Test. (↑) indicates that supernatants from *ex vivo* PBMC cultures and plasma samples have been collected and stored at -80 °C at the respective time points, but cytokine concentrations have not been measured in the present study.

Fig 2. Effects of urban versus rural upbringing on TSST-induced changes in PBMC counts and plasma IL-6 concentrations. Urban compared with rural upbringing was associated with an exaggerated increase in A) the number (#) of viable peripheral blood mononuclear cells (PBMCs) per ml blood and B) plasma interleukin (IL)-6 concentrations in response to the Trier Social Stress Test (TSST). Plasma IL-10 concentrations were undetectable at all time points assessed. Data are presented as mean +/- (A) or + (B) SEM. $*P < 0.05$, $***P \leq 0.001$ versus respective basal (-5 min) group; $^{\#}P \leq 0.05$ versus respective rural group. $^{(\#)}P = 0.063$ versus respective rural group. n.a., not assessed.

Fig 3. Effects of urban versus rural upbringing on TSST-induced changes in *ex vivo* cytokine secretion from isolated PBMCs. Compared with rural participants, urban participants showed unaffected A) basal and B) lipopolysaccharide (LPS), but increased C) concanavalin A (ConA)-induced *ex vivo* secretion of interleukin (IL)-6, both at the -5 min and the 120 min time

point of the Trier Social Stress Test (TSST). Interleukin-10 secretion was undetectable under basal conditions, but lower in both D) LPS and E) ConA-stimulated peripheral blood mononuclear cells (PMBCs) from urban, but not rural, participants assessed at the 120 min time point of the TSST compared with IL-10 values assessed at the -5 min time point of the TSST. Data are presented as mean + SEM. $*P < 0.05$, $***P \leq 0.001$ versus respective basal (-5 min) group; $^{\#}P \leq 0.05$ versus respective rural group.

Fig 4. Effects of urban versus rural upbringing on basal and TSST-induced HPA axis and cardiovascular (re)activity. Urban and rural upbringing were associated with a comparable hypothalamic-pituitary-adrenal (HPA) axis and autonomic nervous/cardiovascular system activation in response to the Trier Social Stress Test (TSST), indicated by comparable increases in a A) plasma cortisol, B) salivary alpha amylase, C) mean arterial blood pressure and D) heart rate. Initial HPA axis activity A) was increased in rural versus urban participants. Data are presented as mean ± SEM. $***P \leq 0.001$ versus respective basal (-5 min) group; $^{\#}P \leq 0.05$ versus respective rural group.

1. Supplementary Sections (SupSec)

1.1 TSST: At the beginning of the test the experimenter guided the participant into the TSST room and positioned him in the center of the room, facing a video camera and a jury consisting of 2 judges sitting behind a table and wearing white lab coats. The judges were told to maintain a neutral evaluative facial expression during the whole test procedure. The experimenter introduced the participant to a standardized job advertisement, for which he wanted him to apply later during the test by explaining why his personality made him ideally qualified for this dream job. After this brief familiarization with the test setting the experimenter guided the test person back into the adjacent room, allowing him to prepare for the simulated job interview for 3 minutes. Before the test person was brought back to the test room, he was asked to complete the Primary Appraisal Secondary Appraisal Scale (PASA), which, on average, took about 2 min. Back in the test room, the participant was asked to start with the public speech, without any information about the intended duration of this speech. In cases of more than 20 sec of silence, the jury started to ask neutral and standardized questions on potential job qualifications of the participant. After 5 min, the experimenter came back and explained the now imminent arithmetic task, consisting of counting backwards from 3079 by subtraction of 17, again not providing information of the intended duration of this task. Whenever failing, the participant was asked to start again at 3079. After 5 min the TSST was finished.

1.2 Blood pressure and heart rate: The cuff placed around the dominant arm at the -60 min time point stayed in place until the last measurement was performed at the 120 min time point; the connection between the cuff and the device was released after each measurement. During measurement of blood pressure and heart rate the participant was sitting on a chair, placing the arm in a slightly bent position on a table.

1.3 PBMC isolation and stimulation: 9 ml blood were transferred from lithium-heparin-coated monovettes into Leucosep™ tubes (Greiner Bio-One GmbH, Frickenhausen, Germany), which were prepared with Ficoll® Paque (GE Healthcare Life Sciences, Freiburg, Germany) according to the manufacturer's instructions beforehand. The remaining volume was filled up to 50 ml with PBS and then centrifuged for 10 minutes at room temperature (1000g, no brake). The buffy coat layer containing PBMCs was transferred into another 50 ml Falcon® tube and washed with RPMI medium containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (323 g, 10 minutes, room temperature). The number of viable (trypan blue) cells was then determined using an automated cell counter (TC20™ Automated Cell Counter, BIO-RAD Laboratories, Munich, Germany), before cells were centrifuged again (323g, 10 minutes, room temperature) and adjusted to a final concentration of 2.5×10^6 cells/ml. 2.5×10^5 cells were then cultured in 96-well plates, either under basal conditions (100 µl RPMI were added to a final volume of 200 µl per well) or in the presence of concanavalin A (ConA; final concentration in 200 µl volume was 2.5 µg/ml) or lipopolysaccharide (LPS; final concentration in 200 µl volume was 1 µg/ml) at 37 °C and 5% CO_2 for 24 hours. Supernatants were collected afterwards and stored at -80 °C until further analysis.

1.4 Enzyme-linked immunosorbent assay (ELISA): Plasma samples were analysed using commercially available ELISA kits for interleukin (IL)-6 (Quantikine HS ELISA, R&D Systems Europe, Ltd.; lowest standard 0.16 pg/ml) and IL-10 (Quantikine HS ELISA, R&D Systems Europe, Ltd.; lowest standard 0.78 pg/ml) and cortisol (IBL International, Hamburg, Germany; lowest standard 20 ng/ml) according to the manufacturers' instructions. Of note, plasma IL-10 concentrations of all participants were under the detection limit of the employed high-sensitive ELISA with the lowest standard being 0.78 pg/ml (Quantikine HS ELISA, R&D Systems, Inc.). Supernatants from PBMC stimulations were analysed using commercially available ELISA Kits (Human DuoSet ELISA, 5 Plate, R&D Systems Europe, Ltd) for IL-6

(lowest standard of 9.38 pg/ml) and IL-10 (lowest standard of 31.3 pg/ml) according to the manufacturer's instructions. Of note, basal *ex vivo* IL-10 concentrations of all participants were under the detection limit of the employed ELISA with a lowest standard of 9.38 pg/ml (Human IL-6 DuoSet ELISA, R&D Systems, Inc.).

1.5 Determination of salivary alpha-amylase concentrations

Salivary alpha-amylase as a surrogate marker of sympathetic nervous system activity was measured as described earlier[28]. In detail, saliva was processed on a FLUENT liquid handling system (Tecan, Crailsheim, Germany). Saliva was diluted at 1:625 with ultrapure water by the liquid handling system. Twenty microliters of diluted saliva and standard were then transferred into 96-well polystyrol microplates (Roth, Karlsruhe, Germany). Standard was prepared from "Calibrator f.a.s." solution (Roche Diagnostics, Mannheim, Germany) with concentrations of 326, 163, 81.5, 40.75, 20.38, 10.19, and 5.01 U/L alpha-amylase, respectively, and ultrapure water as zero standard. Afterwards, 50 µl of substrate reagent (alpha-amylase EPS Sys; Roche Diagnostics, Mannheim, Germany) was pipetted into each well. The microplate containing sample and substrate was then heated to 37 °C in a Thermomixer (Eppendorf, Hamburg, Germany). Immediately afterwards, a first interference measurement was obtained at a wavelength of 405 nm using a standard absorbance reader (Infinite M200, Tecan, Crailsheim, Germany). The plate was then incubated for another 5 min at 37 °C, before a second measurement at 405 nm was taken. Increases of absorbance in samples were transformed to alpha-amylase concentrations using a linear regression computed against the standard curve on each microplate. Inter- and intra-assay variation was below 10%.

2. Supplementary Tables

SupTab 1. Sociodemographic features of the rural and urban groups.

Parameter	Mean ± SEM or percent RURAL	Mean ± SEM or percent URBAN	*P*-value (*t*-test; chi²)
Age (years)	25.05 ± 0.78	24.45 ± 0.88	0.613
Height (cm)	182.8 ± 1.44	182.0 ± 1.49	0.701
Weight (kg)	82.35 ± 1.58	80.75 ± 2.22	0.561
BMI (kg/m²)	24.40 ± 0.65	24.65 ± 0.39	0.748
Marital status			0.147
Married	10%	5%	
Single	90%	95%	
Relationship			0.739
Short-term single	15%	10%	
Long-term single	20%	20%	
Alternating partners	0%	5%	
Long-term relationship (married)	10%	5%	
Long-term relationship (unmarried)	55%	60%	
Children			0.147
Yes	0%	10%	
No	100%	90%	
Education			0.055
General school graduation	10%	0%	
Secondary school without university entrance diploma	25%	5%	
Secondary school with university entrance diploma	65%	95%	
High Education (ISCED)	20%	15%	0.677
Professional qualification			0.251
Still in education	60%	75%	
Apprenticeship	20%	10%	
Apprenticeship with master craftsman´s diploma	10%	0%	
University	10%	15%	
Professional group			0.585
Unskilled worker	0%	5%	
Skilled worker	30%	5%	
Lower professional group	0%	10%	
Middle professional group	5%	0%	
Higher professional group	5%	10%	
Self-employed	5%	0%	
Never worked before	10%	20%	
Unclear	45%	50%	
Professional situation			0.543
Full-time employment	30%	15%	
Part-time employment	0%	5%	

Casual employment	5%	5%	
In training	65%	75%	
Net income per month			0.756
< 400 €	25%	15%	
400 - 1000 €	35%	30%	
1000 - 1500 €	5%	0%	
1500 - 2000 €	15%	15%	
2000 - 2500 €	15%	25%	
3000 - 3500 €	0%	5%	
3500 - 4000 €	5%	10%	
High income (=more than 1500 € net income per month)	35%	55%	0.204
Daily contact with pets and/or farm animals	35%	0%	0.002
Nutrition			0.147
Meat-eating	90%	100%	
Vegetarian	10%	0%	
Alcohol consumption			0.376
Non-drinking	10%	5%	
Less than once a month	15%	25%	
Once a month	0%	10%	
More than once a month	20%	35%	
Once a week	25%	15%	
Two or three days a week	25%	10%	
Nearly daily	5%	0%	

Shown is the mean ± SEM or the percentage of rural/urban participants per group and the *p*-value provided by statistical analysis using either *t*-test or chi² test. Abbreviations: ISCED: International Standard Classification of Education.

SupTab 2. Summary of the results generated by the various questionnaires employed in the present study during recruiting, as well as before and after TSST exposure.

Parameter	RURAL	URBAN	*P*-value (*t*-test; chi²)
STAI-S	**Mean ± SEM**	**Mean ± SEM**	
Before TSST	33.7 ± 1.18	29.85 ± 0.92	0.014
After TSST	33.05 ± 1.34	27.75 ± 1.19	0.005
BL	**Mean ± SEM**	**Mean ± SEM**	
Complaints	5.95 ± 1.32	4.3 ± 0.99	0.324
PASA	**Mean ± SEM**	**Mean ± SEM**	
Threat	3.56 ± 0.26	2.68 ± 0.16	0.005
Challenge	4.59 ± 0.14	4.03 ± 0.21	0.032
Self-concept of own abilities	3.93 ± 0.27	4.28 ± 0.19	0.299
Locus of control	4.61 ± 0.19	4.64 ± 0.15	0.919
Primary appraisal	4.08 ± 0.16	3.36 ± 0.16	0.004
Secondary appraisal	4.27 ± 0.18	4.46 ± 0.11	0.388
Stress index	-0.19 ± 0.29	-1.08 ± 0.24	0.025
HADS-D	**Mean ± SEM**	**Mean ± SEM**	
Anxiety	4.25 ± 0.6	2.65 ± 0.35	0.027
Depression	2.15 ± 0.36	2.45 ± 0.49	0.626
PSS-4	**Mean ± SEM**	**Mean ± SEM**	
Stress scale	5.1 ± 0.58	4 ± 0.52	0.165
CTQ	**Mean ± SEM**	**Mean ± SEM**	
Emotional abuse	6.45 ± 0.64	5.58 ± 0.18	0.209
Physical abuse	6.45 ± 0.46	5.6 ± 0.28	0.125
Sexual abuse	5 ± 0	5 ± 0	1.000
Emotional neglect	9.6 ± 0.72	7.9 ± 0.66	.089
Physical neglect	6.1 ± 0.29	5.5 ± 0.22	.109
CECA-Q	**Mean ± SEM**	**Mean ± SEM**	
Maternal aversion	14.1 ± 1.54	11.45 ± 0.84	.140
Maternal neglect	12.4 ± 1.26	12.2 ± 1.27	.912
Paternal aversion	15.9 ± 1.34	14.35 ± 1.12	.380
Paternal neglect	16.65 ± 1.49	16.15 ± 1.46	.812
SCID-I (Telephone screening)	**No/Unclear/Yes** in %	**No/Unclear/Yes** in %	
Alcohol (Times with more than 5 drinks at one occasion?)	20/0/80	15/0/85	0.678
Drugs (Ever taken?)	55/5/40	45/10/45	0.744
Pharmaceuticals (Felt dependent on or took more than prescribed?)	100/0/0	100/0/0	n.a.
Panic attacks (Ever experienced?)	100/0/0	100/0/0	n.a.
Agoraphobia (Ever experienced?)	100/0/0	100/0/0	n.a.
Social anxiety (Ever experienced?)	100/0/0	100/0/0	n.a.
General anxiety (Ever experienced?)	95/0/5	90/0/10	0.548
Compulsive thoughts (Ever experienced?)	100/0/0	100/0/0	n.a.
Compulsive acts (Ever experienced?)	100/0/0	100/0/0	n.a.

Particularly nervous or anxious (During last 6 months?)	85/5/10	60/25/15	0.155
Extraordinarily lean (Ever mentioned by others?)	90/0/10	100/0/0	0.147
Binge eating (Ever occurred?)	100/0/0	100/0/0	n.a.
SCID-I (Affective part)	**No** in %	**No** in %	
Current Major Depression episode (Questions A1, A2)	100	100	n.a.
Previous Major Depression episode (Questions A38, A39)	100	100	n.a.
Current manic episode (Question A55)	100	100	n.a.
Previous manic episode (Question A90)	100	100	n.a.
Current dysthymia (Question A121)	100	100	n.a.

Shown is the mean ± SEM or percentage of rural/urban participants per group and the *p*-value provided by statistical analysis using either *t*-test or chi² test. Abbreviations: BL: List of complaints for quantitative analysis of current bodily and general complaints; CECA-Q: Childhood Experience of Care and Abuse Questionnaire; CTQ: Childhood Trauma Questionnaire; HADS-D: Hospital Anxiety and Depression Scale-German Version; IL: interleukin; PASA: Primary Appraisal Secondary Appraisal Scale; PBMC: peripheral blood mononuclear cell; PSS-4: Perceived Stress Scale-4; SCID-I: Structured Clinical Interview for DSM-IV Disorders; STAI-S: State(-Trait-)Anxiety-Inventory; TP: time point; TSST: Trier Social Stress Test.