Lifestyle Factor Stress and Atherosclerosis

Studies on myeloid cell topography and migration

Agnes A. Mooslechner

Immunology PhD Candidate

Marshall Plan Scholarship Recipient

August 2019 – April 2020

Clinical Division of Nephrology

Department of Internal Medicine

Medical University of Graz

Austria

Center for Systems Biology

Massachusetts General Hospital and

Harvard Medical School

USA

Table of Contents

Introduction

Stress is a universal phenomenon of our time. However, the society's perception of stress has dramatically changed within the last decade. While just a few years ago the stereotype of an overworked ever busy persona was celebrated, today millions in revenue are generated with meditation apps, awareness courses and stress relief products [1]. We came to realize, that next to detrimental effects on mental health, stress is associated with serious physical implications. Chronic stress symptoms include headaches, dizziness, general aches and pain, grinding teeth, clenched jaw, indigestion or acid reflux symptoms, increase or loss of appetite, muscle tension, problems sleeping, racing heart, exhaustion, trembling and an upset stomach [2]. However, the underlying mechanisms causing these symptoms are not well understood. An approaching deadline is not an immediate threat to our body's integrity. So how can a solely psychological stressor unleash such powerful alterations to our physical health?

In 1915 Walter Bradford Cannon noted that our body responds to stress in order to mobilize great energy and thereby improving our potential to escape or attack. Cannon called his observations the "fight-or-flight" response [3]. In 1936 Hans Selye defined three stages of stress: the alarm phase with acute manifestations, the resistance phase in which the acute manifestations disappear and the exhaustion phase, when the first phase may be present again or the organism may collapse due to phase one and two [4]. Selye was the first scientist to define stress from a biological point of view as "a nonspecific response of the body to any demand made upon it" [5]. He distinguished between positively perceived stress and negatively perceived stress, termed eustress and distress [6]. Today however the concept of eustress and distress is disputed among the scientific community. It is thought that stress cannot be intrinsically good or bad and that the adaptation to it, depends on various environmental and internal factors (e.g. the history of such encounters or active disease) [7]. Even though, the initial concepts of stress have been revised and extended in the last decades, they are in general still accepted today.

We know, that the body's stress response is conserved, highly efficient and complex. It is triggered upon perception of a potential threat (also known as stressor) with the goal to maintain physiological integrity even in the most adverse conditions. Simply put, stressors lead to the release of mediating molecules which allow communication between the brain and the periphery and vice versa. This process is called the stress response, which through physiological and behavioral mechanisms eventually restores homeostasis in the body and additionally promotes adaptation [8].

When talking about stress, we need to distinguish between psychological and physical stressors. These two types of stressors are processed by different circuitries in the brain, therefore engage in distinct neuronal networks and result in different cellular activity, which may overlap in some instances. Psychological stressors are caused by aversive environmental stimuli such as predator-related cues or the failure to satisfy internal drives, like not being able to move freely. The brain responds to such stimuli based on prior experience and responses can already be triggered in anticipation. Fear as a stimulatory input is processed by very specific brain regions, one of them being the amygdala. The amygdala complex can be divided into the basolateral nucleus (BLA), the central nucleus of the amygdala and the medial nucleus. The BLA has a major role in processing psychological stressors and can be significantly triggered by anticipatory stressors and is therefore fundamental in detecting potential danger [9]. On the other hand, blood loss, injuries or infections are physical stressors, which require rapid systemic reactions, which might be considered reflexive. Physical stressors are mainly processed by brainstem and hypothalamic regions.

The most rapid response to stressors is mediated by the autonomic nervous system (ANS). Its sympathetic and parasympathetic arms can act on end organs via neural innervations, causing rapid alterations in physiological states. Through the sympatho-adrenomedullary (SAM) arm the ANS can quickly excite the cardiovascular system by increasing the heart rate and blood pressure [10]. But these effects wane as quickly as they are induced. The rapid physiological adaptation is mainly mediated by catecholamines. Adrenaline and noradrenaline are secreted from the adrenal medulla and partly from sympathetic nerves [8,11,12]. Responsible for the autonomic modulations are projections from the paraventricular nucleus of the hypothalamus (PVN), the locus coeruleus (LC) and the rostral ventrolateral medulla (RVLM) to pre-ganglionic sympathetic neurons present in the thoracolumbar region of the spinal cord. In response, the adrenal medulla synthesizes and secretes adrenaline and noradrenaline. These two components are key in sympathetic system activation, which evoke changes in blood vessels, glands, visceral organs and smooth muscles [12]. The increase of adrenaline and noradrenaline levels prepare the body for the “fight-or-flight” reaction. The effects include alertness, increase of metabolic actions and increase of the cardiovascular capacity.

The parasympathetic component of the ANS regulates the duration of autonomic responses of the cardiac and respiratory systems. The pre-ganglionic parasympathetic neurons originate from the brainstem and sacral spinal cord with post-ganglionic neurons usually in the wall of the target organs. Stimulation of the respective receptors on target cells is mediated by acetylcholine [13]. The sympathetic and parasympathetic systems act independently from each other, with the parasympathetic system dominating during resting conditions only.

The Hypothalamic-pituitary-adrenocortical (HPA) axis initiation is only relatively slower compared to the ANS. It results in an increase of circulating glucocorticoid levels with peak levels occurring just minutes after triggering the response, leading to short- and long-lasting physiological effects. The HPA axis is activated in a coordinated sequence. The PVN synthesizes oxytocin, vasopressin and the corticotropin-releasing-hormone (CRH), which stimulate the pituitary gland to produce and release adrenocorticotropin (ACTH). ACTH is secreted and acts on the cortex of the adrenal gland, which triggers glucocorticoid synthesis and secretion [14,15]. In humans the main glucocorticoid is cortisol, which can be also found in rodents as corticosterone. Glucocorticoids are steroids and can reach any cell in the body and act on a variety of target organs by binding to glucocorticoid receptors, which can be found on numerous cell types.

Glucocorticoid receptors form the connection between the endocrine system and the immune system, explaining the relationship between stress hormones and immunological changes. These findings have highlighted many pathways which can also be explored for clinical implications. With the development and commercial production of cortisol, a variety of inflammatory disorders like rheumatoid arthritis were suddenly treatable. Stress can directly influence immune signaling by reducing the inhibitory effects of glucocorticoid actions, or by stimulating the HPA axis and SAM [16,17].

Besides the upregulation of pro-inflammatory cytokines, there is more and more evidence that stress has an effect on immune cell dynamics. A study in mice subjected to a model of repeated social defeat showed increased myelopoietic output of Ly-6C^{high} monocytes and Ly-6C^{intermediate} granulocytes. These effects were blocked by pharmacologic antagonists of β-adrenoreceptors and the myelopoietic growth factor GM-CSF. In humans with low socioeconomic status the authors found a selective up-regulation of immature pro-inflammatory CD16^{-} monocytes [18]. Another study showed that chronic stress changes hematopoietic stem cell activity. In mice subjected to a model of chronic variable stress, higher levels of noradrenaline were detected in the bone marrow. This increase was associated with a decrease in mRNA and protein levels of retention-associated CXCL12. Noradrenaline binds to β_3-adrenergic receptors on niche cells, which results in downregulation of CXCL12. Subsequently, hematopoietic stem cell proliferation was elevated. Mechanistically, the authors found increased cycling of Lin^{-}Sca-1^{+}c-Kit^{+}CD150^{+}CD48^{-} hematopoietic stem cells, which also incorporated a higher amount of BrdU. Increased numbers of neutrophils and monocytes were measured in the periphery. The elevated output of proinflammatory cells was correlated with factors positively associated with myocardial infarction. In a mouse model of atherosclerosis, flow cytometry analysis revealed higher numbers of neutrophils, macrophages and monocytes in blood of stressed animals [19].

The cytokine expression profile showed increased levels of myeloperoxidase but the plaque size was not changed in stressed atherosclerotic mice. Inflammatory monocyte and macrophage infiltration of atherosclerotic plaques correlated with plaque rupture and increased mortality [20]. Chronic stress induced neutrophilia and monocytosis was also found in humans [19].

B lymphocytes also seem highly affected in their dynamics by stress exposure. A number of studies show impairment of antibody response to vaccination under chronic stress conditions and elevated levels of glucocorticoids [21–26]. In mice, high levels of glucocorticoids were associated with a 50% reduction in peripheral blood B lymphocytes. Garvy et al. explained these shifts by apoptosis of pro-B, pre-B and immature B lymphocytes in the bone marrow [27]. Later studies confirmed that early human B cells are highly sensitive to glucocorticoids, while mature IgD^+ B cells are fairly resistant to glucocorticoid induced apoptosis [28,29]. Low levels of $CD19^+$ B lymphocytes and a flattened cortisol awakening response were associated with perceived stress among graduate students.

Stress mediators operate in a feedback loop and regulate and restore homeostasis. Predicting the significance of environmental stressors is crucial for an appropriate physiological response. Excessive responses to harmless stressors are metabolically costly. However, the underestimation of threats can be lethal. Stressful situations and wrong estimations of non-serious threats are mentally exhausting and clearly have an effect on our immune system. The continuing exposure to stress is correlated with a large number of comorbidities [30]. In everyday life, experiencing positive outcomes to stressful events allow us to adapt our stress response and to keep calm if we are exposed to the same stressor again. In our study, we focus on the question if stress adaption is also possible on a cellular level.

Results

Acute Stress induces neutrophil swarming from the bone marrow

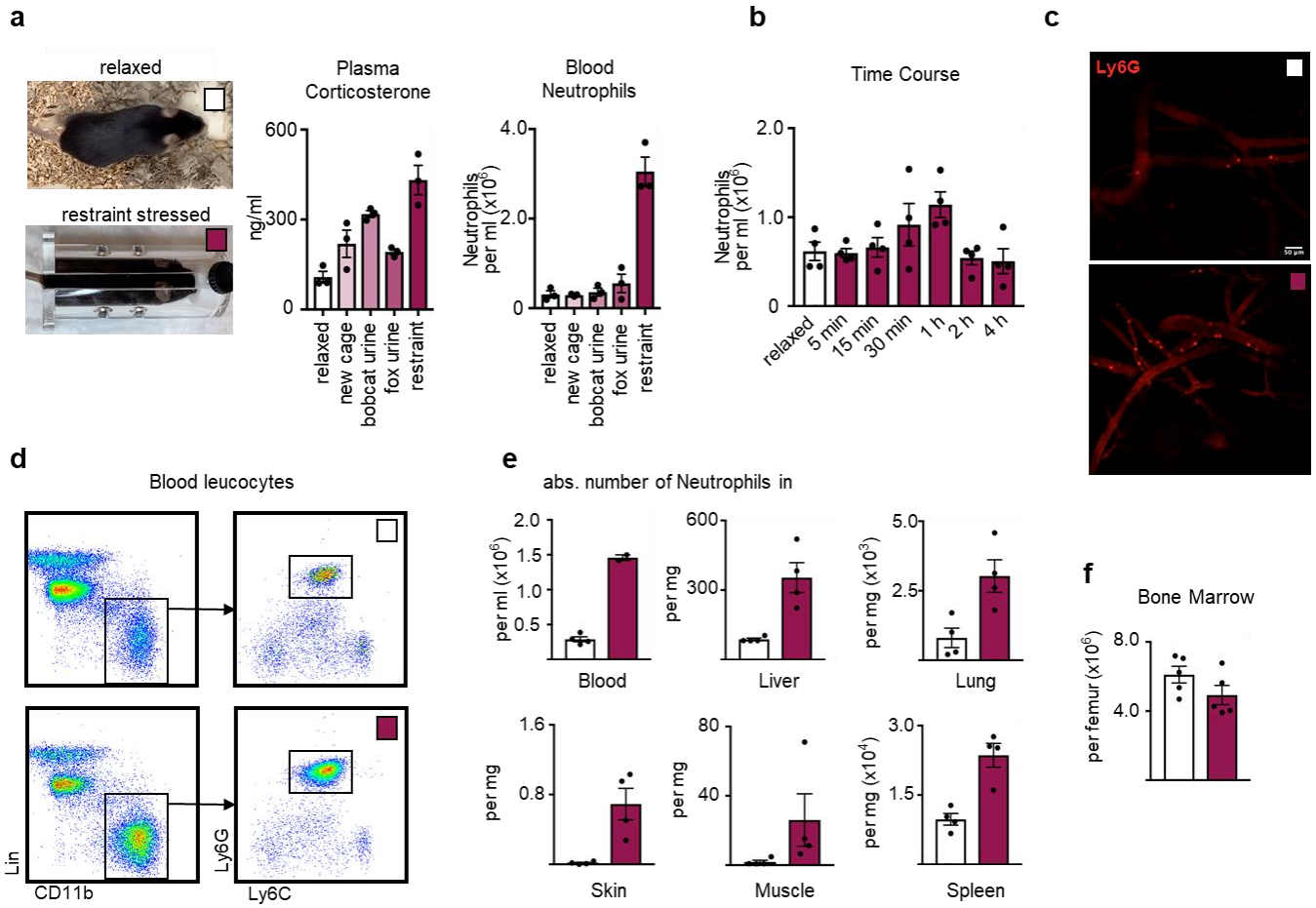

Fig.1: Strong acute stressors trigger neutrophil migration from the bone marrow to the periphery in an exposure time-dependent manner. Assessment of acute stress in male C57BL/6J mice. **a**, Quantification of plasma corticosterone levels and absolute numbers of blood neutrophils in single-housed mice (n=3 per group) exposed to different kinds of stressors. **b**, Time course of blood neutrophilia induced by restrain stress (n=4 per group). **c**, Example of in vivo visualization of $Ly6G^+$ cells in the small vasculature of the ear of relaxed (white) vs. stressed mice (red). **d**, Representative flow cytometry dot plots of $CD11b^+Ly6G^+$ double positive cells in relaxed vs. stressed mice. **e**, Quantification of absolute neutrophil numbers after 1 hour of restrain stress in blood, liver, lung, skin, muscle, spleen (n=2-4 per group) and **f**, bone marrow (n=5per group).

To establish a model of acute psychological stress we subjected male C57Bl/6J mice to different stressors (Figure 1a). Placing mice in new cages or exposure to bobcat or fox urine resulted in elevated corticosterone levels. The highest increase in corticosterone was detected in mice subjected to restrain stress in transparent cylinders, which was also reflected in a 10-fold increase of peripheral neutrophils in blood. No other tested psychological stressor was able to trigger neutrophil swarming to the periphery compared to a relaxed control group.

In a time-course experiment, we found the highest number of neutrophils circulating in the blood in mice restrained for 1 hour. Shorter and longer restrain-time resulted in lower numbers of neutrophils in the periphery (Fig. 1b).

Intra vital microscopy confirmed flow cytometry data. Animals that were stressed for 1 hour showed more circulating Ly6G positive cells in the microvasculature of the ear in vivo (Fig. 1c). To investigate whether this phenotype is exclusive to the vasculature of stressed mice, we analyzed a broad spectrum of tissues via flow cytometry (Fig. 1d and e). As seen in blood, absolute neutrophil numbers in liver, lung, skin, muscle and spleen of animals exposed to an acute psychological stressor (restraint stress) were dramatically increased compared to relaxed controls.

The largest pool of neutrophils is known to reside in the bone marrow. Stressed mice show a reduction in neutrophil numbers in this pool, which suggests a stress-induced swarming from the bone marrow to the periphery (Fig. 1f).

Neutrophil swarming upon stress is mediated by the sympathetic nervous system

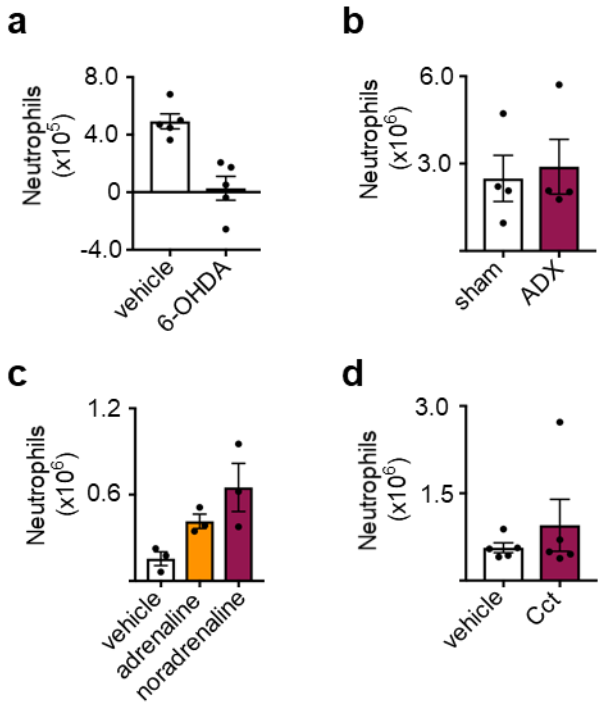

Fig.2: The sympathetic nervous system controls neutrophil swarming upon stress. Assessment of acute stress in male C57BL/6J mice. **a**, Change in absolute blood neutrophil numbers in mice treated with vehicle and sympathectomized with 6-OHDA (n=5 per group). **b**, Quantification of blood neutrophils per ml in sham operated vs adrenalectomized mice (n=4 per group). **c**, Quantification of neutrophils per ml blood of non-stressed mice injected with adrenaline or noradrenaline (n=3 per group). **d**, Absolute neutrophil numbers per ml blood of non-stressed mice injected with corticosterone or vehicle (n=5 per group).

The sympathetic nervous system plays a crucial part in the body's response to stress. The key mediators of the SNS are catecholamines. To investigate whether the SNS is responsible for the increase of neutrophils in the periphery of stressed mice, male C57Bl/6J mice were injected with 6-hydroxydopamine (6-OHDA). This synthetic neurotoxin enters the noradrenergic neurons in the periphery via noradrenaline reuptake transporters. 6-OHDA is used to selectively destroy noradrenergic neurons, which resembles SNS ablation. Upon exposure to stress, vehicle treated mice showed a strong increase in blood neutrophils, whereas this increase was almost completely blunted in the 6-OHDA treated mice (Fig. 2a).

The body's main sources of circulating adrenaline and noradrenaline are the adrenal glands. To test the hypothesis that circulating catecholamines function as regulators of stress-induced neutrophil swarming, mice were subjected to bilateral adrenalectomies (ADX) or a sham procedure. Upon stress, both groups showed similar increases in circulating neutrophils, suggesting that the SNS might act locally in the bone marrow to trigger swarming (Fig. 2b).
On the other hand, relaxed mice injected i.p. with adrenaline or noradrenaline showed higher levels of neutrophils in their blood (Fig. 2c).

In contrast to catecholamine injections, corticosterone injections did not induce neutrophilia (Fig. 2d). Overall, these results suggest that stress-induced neutrophil swarming is initiated by the SNS on a local bone marrow level dependent on catecholamines but independent of corticosterone and therefore the HPA-axis.

Acute stress induced neutrophilia prepares the body for upcoming injury

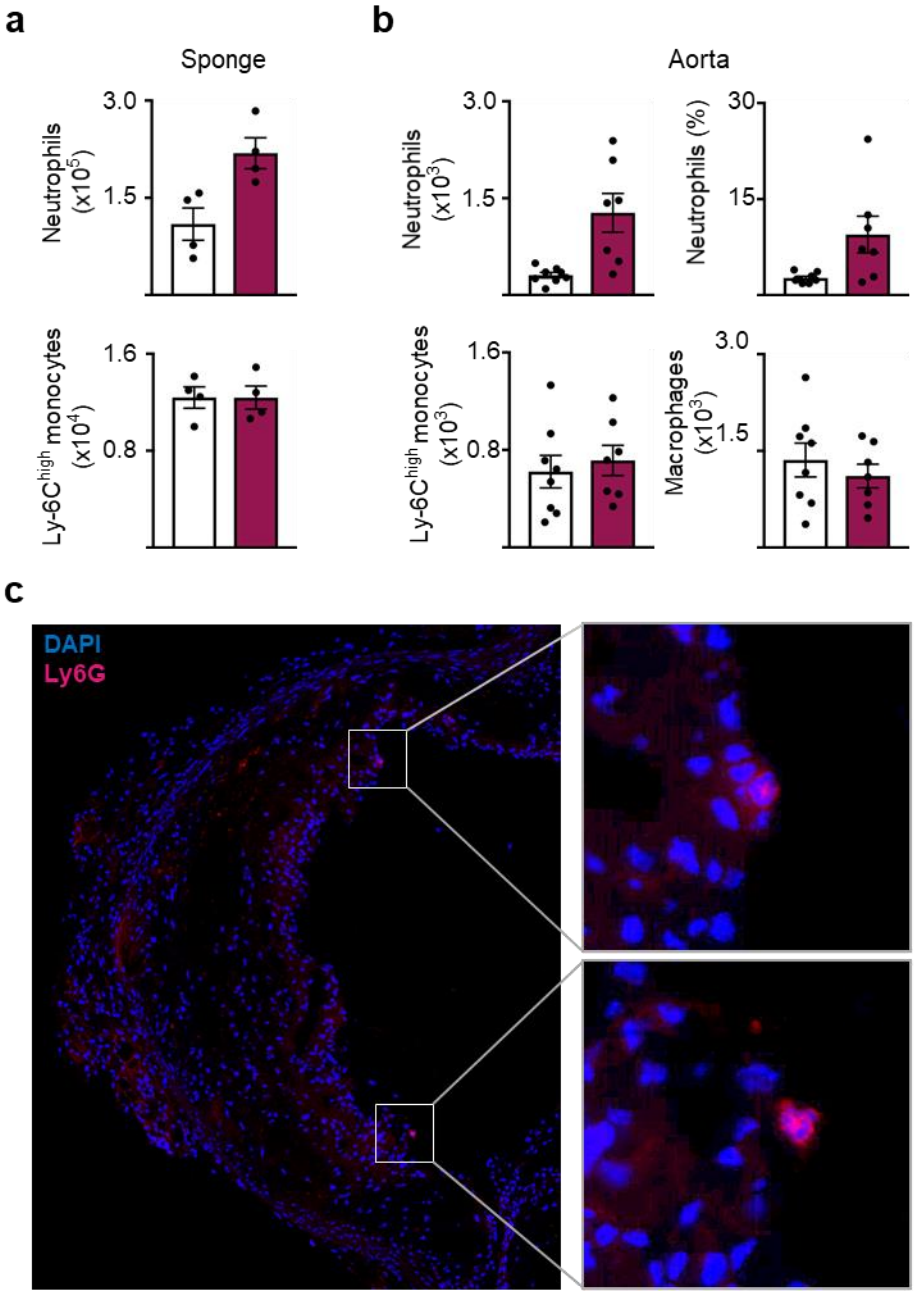

Fig.3: The double-edged sword of acute stress induced neutrophilia. Assessment of acute stress induced neutrophil swarming in an injury model in C57Bl6/J mice and atherosclerosis model in Apoe-/- mice on high fat diet. **a**, Quantification of neutrophils and Ly-6C^{high} monocytes in subcutaneous surgical sponge implants after acute stress (n=4 per group). **b**, Quantification of neutrophils, Ly-6C^{high} monocytes and macrophages as well as neutrophil frequencies in aortas of relaxed vs stressed Apoe-/- atherosclerotic mice (n=7-8 per group). **c**, Representative immunofluorescent staining of a plaque in the aortic root of a stressed Apoe-/- mouse stained with DAPI and Ly6G antibodies. Magnification of Ly6G positive cells in shoulder regions of the plaque's fibrous cap.

We then focused on possible functions of neutrophil swarming. Neutrophils play a role in bacterial clearance and are therefore crucial in the defense to infection. To test whether stress-

induced peripheral neutrophil swarming is a measure of the body to prepare the immune system for upcoming injuries, pre-stressed animals were subcutaneously implanted with surgical sponges. Flow cytometric analysis of these sponges 18 h later revealed significantly higher numbers of neutrophils, but not monocytes, in the sponges of mice subjected to acute psychological stress directly before the injury (Fig. 3a). These data suggest that psychological stressors alert the body and prepare it for expected injuries.

In turn, however, stress is also known to aggravate several diseases. Atherosclerotic plaques are shown to be less stable with growing numbers of infiltrating neutrophils. We found that atherosclerotic Apoe$^{-/-}$ mice on high fat diet indeed have higher numbers of neutrophils in their aortas upon acute stress (Fig. 3b). Monocyte and macrophage numbers were comparable in relaxed and acutely stressed mice. Fluorescence microscopy revealed that neutrophils reside in the shoulder regions of the fibrotic caps, where they may contribute to plaque erosion and rupture (Fig. 3c).

Stress training prevents acute stress-induced neutrophilia

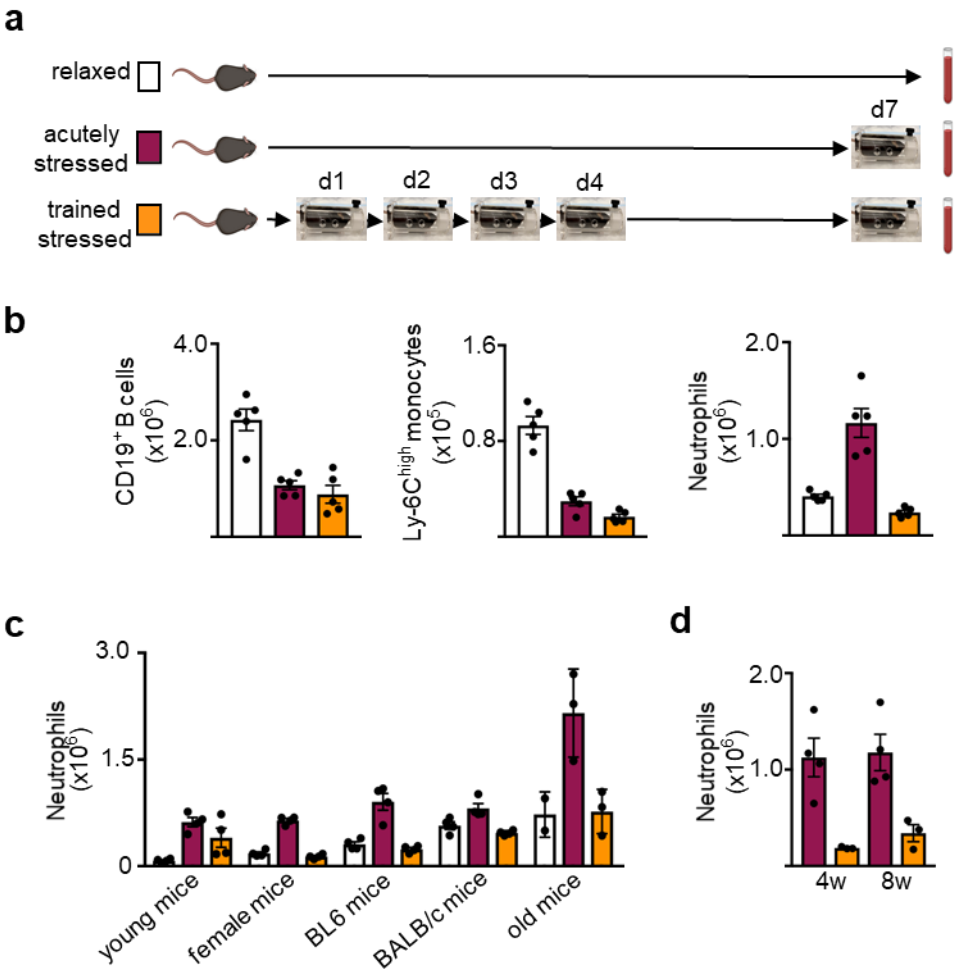

Fig.4: Stress-induced neutrophilia is a universal phenomenon which is trainable. Assessment of first-time stress exposure vs re-exposure to the trained stressor in C57BL/6J mice. **a**, Illustration of stress training experiments. Mice were stressed daily for 4 days and on day 7 as readout. Results were compared to relaxed controls and first-time stress exposure (n=5 per group). **b**, Quantification of $CD19^+$ B cells, Ly-$6C^{high}$ monocytes and neutrophils in mice upon re-exposure to trained stressor vs controls. **c**, Quantification of neutrophils in blood of young, old, female and male C57Bl6/J mice and BALB/c mice (n=3-4 per group). **d,** Peripheral neutrophil numbers in mice upon re-exposure to trained stressor in mice 4 and 8 weeks after stress-training (n=3-4 per group).

To investigate whether the body can adapt to the immunological effects of acute psychological stress, we subjected mice to stress periods on 4 consecutive days (Fig. 4a), subsequent referred to as stress-training. At re-exposure to the psychological stressor, $CD19^+$ B cells and Ly-$6C^{high}$ monocytes drop drastically in numbers and therefore behave the same way as seen in a control group which was exposed to the stressor for the first time. In strong contrast to the unchanged response of B cells and monocytes, the neutrophil increase seen during first-time stress exposure was completely blocked when mice were re-exposed to the trained stressor (Fig. 4b). These training effects could already be seen after one preceding training episode

before read out (data not shown). Additionally, the first-time exposure and training effects of psychological stress on neutrophils turned out to be a universal phenomenon. The same pattern of an increase upon first time stress exposure and a blunted response in animals re-exposed to the trained stressor was observed in male and female as well as in young (4 weeks) and old (18 months) C57Bl/6J mice, and additionally also in male BALB/c mice which represent a different mouse strain (Fig. 4c). To determine the duration of the neutrophil stress training effect, we subjected groups to four training episodes followed by 4 or 8 weeks of standard husbandry with no stressor exposure. Upon re-exposure, both stress-trained groups still showed no signs of neutrophilia, when compared to groups experiencing first time stress (Fig. 4d).

Effects of stress-training are mediated by alterations of the bone marrow niche

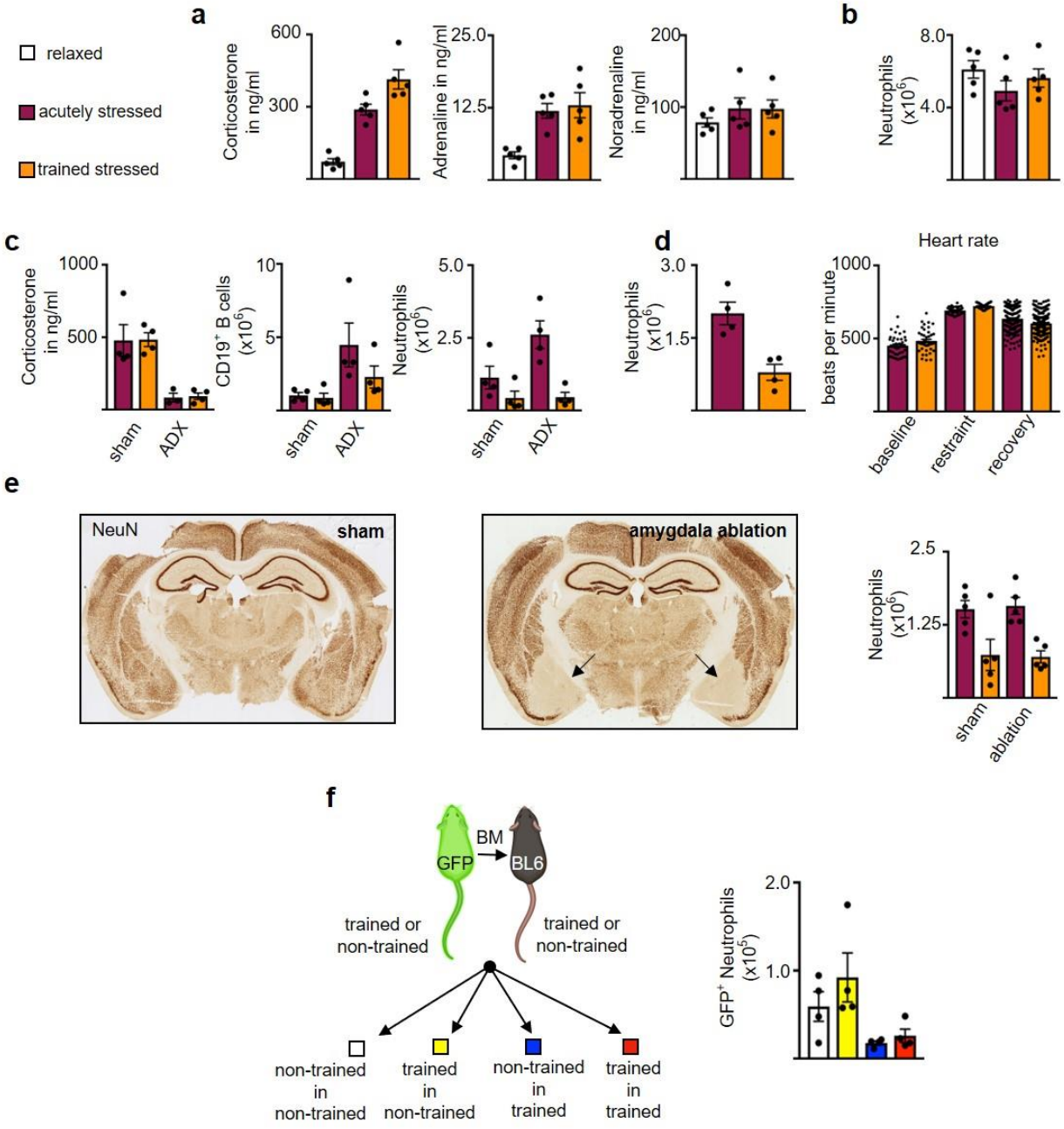

Fig.5: The effects of stress-training are mediated by the bone marrow niche and not by psychological adaption to the stressor. Assessment of stress-related parameters in stress-trained mice. **a**, Stress-trained mice show similar or even higher levels of corticosterone, adrenaline and noradrenaline compared to mice which were exposed to the stressor for the first time (n=5 per group). **b**, The bone marrow neutrophil pool is not depleted in stress-trained animals (n=5 per group). **c**, Stress-trained adrenalectomized mice have comparable corticosterone levels, but lower CD19$^+$ B cells and neutrophils than first time stress-exposed mice (n=4 per group). Acute stress-induced neutrophilia as well as the stress-training effects are independent of adrenal glands. **d,** Comparable heart rates in beats per minute of stress-trained mice vs. first time stress exposed mice (n=4 per group), despite stress mediated differences in neutrophil numbers. Heart rate data covers the baseline, restrain stress period and recovery phase. **e,** NeuN staining of sham injected and amygdala ablated mice. Quantification of neutrophils of amygdala ablated or sham operated mice, either exposed to the stressor for the first time or stress-trained mice (n=5 per

group). Amygdala ablation does not prevent stress-induced neutrophilia nor the effects of stress training. **f,** Whole bone marrow transfer of stress-trained or non-trained GFP-Ubi mice in stress-trained or non-trained C57Bl/6J mice (n=4 per group). Quantification of non-trained or trained GFP+ peripheral neutrophils upon stress exposure in trained or non-trained recipients. The occurrence of neutrophil swarming depends on the training status of the bone marrow niche of the recipient.

In search for the mechanisms behind this phenomenon, we investigated whether the stress training effect on neutrophils is explainable by a conscious adaptation of the mouse to the stressor. We analyzed parameters of stress in groups experiencing first-time stress vs. re-exposure to the trained stressor. Stress was measured via plasma levels of corticosterone, adrenaline and noradrenaline, which were comparable in both groups (Fig. 5a). In fact, mice trained to the stressor showed even higher levels of corticosterone. The neutrophil pool in the bone marrow was not depleted after repeated stress episodes (Fig. 5b). To investigate whether the effect depends on corticosterone, adrenal glands of two groups were surgically removed. Mice were either exposed to the stressor for the first time or re-exposed to the trained stressor. Two groups of sham operated mice were stressed accordingly. Results showed that upon adrenalectomy, corticosterone levels were negligibly low, resulting in a rescue of the drastic stress induced decrease in B cell number in both groups (Fig. 5c). However, the absence of corticosterone even tended to increase the acute stress induced neutrophilia with no loss of the training effect.

An increased heart rate is an indicator of the level of stress and sympathetic activity. To test whether stress-trained mice are simply not as stressed as when exposed to the stressor for the first time, two groups of mice were equipped with telemeters capable of measuring the heart rate. Upon stress, the stress-trained group showed lower levels of neutrophils, but the same heart rate changes from baseline to stress episode and in the recovery period compared to a group exposed to restrained stress for the first time (Fig. 5d). The amygdala is an important fear center of the brain. Humans lacking the amygdala, among other complications, experience no fear, making this cluster of nuclei crucial for fear and stressor adaptation. We chemically ablated the amygdala as seen representatively in Fig. 5e. Stress induced neutrophilia was independent of amygdala ablation. Additionally, the amygdala also had no impact on the capability to blunt the increase in neutrophils in stress-trained mice.

Next, we wanted to know if the training effect is mediated by an adaptation of neutrophils or their progenitors themselves or rather by changes in the bone marrow niche microenvironment. Therefore, we performed adoptive transfers of GFP+ bone marrow cells into wild type mice. We either transferred bone marrow of stress-trained or non-trained GFP+ donor mice into stress-trained or non-trained wild type recipients. The results showed that neutrophils are able

to swarm the periphery upon psychological stress regardless of training status in non-trained recipients. However, transferred neutrophils were not detected in the periphery and were likely retained in the bone marrow of stress trained recipients, regardless of their own training status (Fig. 5f).

Stress-training is beneficial in atherosclerosis and does not disturb the neutrophil response in infection or injury

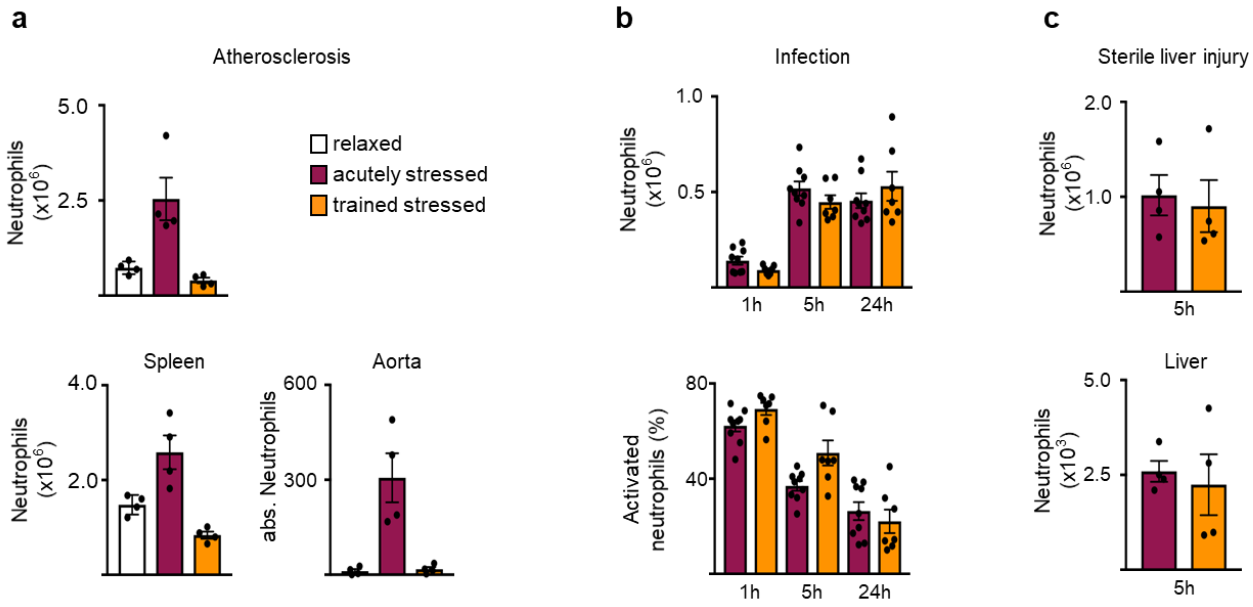

Fig.6: The training-mediated altered stress response is beneficial in atherosclerosis, but effects are overwritten in infection or injury. Assessment of stress training vs. first time stress exposure in atherosclerotic Apoe-/- mice and in an LPS-induced infection model as well as a sterile injury model in C57Bl6/J mice. **a**, Quantification of neutrophils in peripheral blood, spleen and aortas of atherosclerotic mice (n=4 per group). **b**, Quantification and activation of peripheral neutrophils in an LPS-induced infection model (n=7-8 per group). **c**, Quantification of neutrophils in blood and liver in a model of sterile liver injury (n=4 per group).

To investigate the disease relevance of stress training, we tested different mouse models of disease. In atherosclerotic mice, stress training lead to lower neutrophil numbers in blood, spleen and thoracic aorta upon re-exposure to the trained stressor (Fig. 6a). However, in an LPS model of infection, the training effect was overruled and neutrophils swarm the periphery in a comparable manner to non-trained mice (Fig. 6b). The same was true for a sterile injury model. Neutrophils werereleased in the occurrence of sterile injury to the liver regardless of prior experienced stress episodes (Fig 6c).

Discussion

We are able to adapt to our dynamic environment and the challenging life events we face. The stress system engages our brain and body to enhance performance and promote adaptation. Stress research is over eight decades old and there are numerous rodent models of psychological stress described in the literature. Stress is reflected in higher cardiac output (e.g. heart rate), corticosterone and catecholamine release. On a cellular level, several studies show that upon stress exposure peripheral B cell levels decrease, while some report neutrophilia [31,32]. Our study identifies a novel mechanism explaining how acute psychological stress may trigger cardiovascular events and how adaptation to psychological stressors is mediated on a cellular level.

A big part of psychological stress models in rodents mirrors a naturalistic survival threat. Water and food deprivation or deprivation of rapid eye movement sleep are well described. Other models focus more on fear-based concepts, like exposure to predator odor or social conflict. In a colony of domesticated laboratory animals, disruption of hierarchic relationships, overcrowding or long-term social isolation are a major source of stressful stimuli [33,34]. To study psychological stress, it is important to draw the line at stress stimuli, which actually induce injury or pain (e.g. foot shock). In our study we tested a new cage environment, predator odor exposure and restraint stress as solely psychological stressors. All methods induced stress in animals, measurable in increased plasma corticosterone levels. However, only restraint stress resulted in time-dependent peripheral neutrophilia. We found that after one hour of stress exposure, neutrophils are detectable in high numbers in most organs and tissues (liver, lung, skin, muscle, spleen), while numbers in the bone marrow decrease. The bone marrow reserve consists of the largest mature neutrophil pool in the body, which were shown to be recruited upon inflammation [32]. Our data indicate that also solely upon acute psychological stress, neutrophils are mobilized from the bone marrow pool and swarm the periphery, resulting in a similar pattern as seen in infection or injury.

The anticipation of a threat is enough to trigger this enormous cell migration. The relationship between sensory input in the brain and connection to the immune system takes place in part by the sympathetic nervous system and the HPA-axis. We tested which pathway mediates the mobilization of neutrophils upon stress. Peripheral ablation of the SNS resulted in dampened neutrophil swarming of the periphery. Treatment with catecholamines showed that neutrophilia is mainly dependent on noradrenaline. Since removal of the adrenal glands did not blunt the stress-induced neutrophilia, it is very likely that local action of the SNS in the bone marrow is responsible for the neutrophil release. On the other hand, we could show that injections of

corticosterone did not result in higher neutrophil numbers, suggesting that the swarming of neutrophils upon stress is independent of the HPA-axis.

As previously mentioned, neutrophilia is a hallmark of wound healing. Neutrophils are recruited to wounds primarily to fight infections. In our study, we could show that psychological stress accelerates the recruitment of these important cells to sites of injury. The accelerated recruitment in response to stress was specific to neutrophils, whereas no difference in Ly6C^{high} monocyte numbers were detected. We hypothesize that neutrophil swarming triggered by anticipation of a possible threat alone is a measure of the body to be prepared for upcoming injuries. Rapidly released mature neutrophils may form a first line of defense. Upon receiving an alert signal which causes a disruption of homeostasis, they circulate the periphery to detect wounds and possible harmful microbial intruders, even before pain or an actual threat has been experienced. Although this rapid machinery is undoubtedly useful, elevated neutrophils may also be detrimental in some instances. The presence of neutrophils in aortic plaques is positively correlated with plaque rupture and therefore with greater risk for myocardial infarction [35–37]. Indeed, there is epidemiological evidence for an association of acute stress and cardiovascular events (e.g. soccer world championships [38]). Our data are in line with the literature and show that exclusively neutrophils are released upon acute psychological stress and that these neutrophils accumulate in the fibrous caps of aortic plaques, which may contribute to destabilization and possibly trigger erosion or rupture.

Resilience to stress is thought to be one of the key factors to success in life, may it be work-, sports- or socially-related. Resilience is the ability of a system to withstand changes in its environment and still function. In our context, resilience can be seen as the adaptation to stress. We were interested if our model of solely psychological stress shows a training effect in our animals. Are they capable of realizing that restraint stress is not a life-threatening scenario? And on which level are they able to adapt? We therefore created a stress training model, where we exposed animals to one hour of restrained stress per day for a period of four consecutive days. After a recovery phase, we re-exposed the animals to the trained stressor and compared the results to a group which experienced the stressor for the first time. We found that CD19^{+} B cells and monocytes decrease in animals in the same manner, regardless of stress training. Interestingly however, we did not see neutrophilia in mice which were re-exposed to the trained stressor. The rapid release of neutrophils is metabolically costly and the system seems to be able to adapt and conserve energy. Why other cell types do not seem to adapt is not clear. It might be that their role in recovering to homeostasis is too important or the processes involved are just not as flexible. The effects of stress training, however, seems conserved and universal. The effects were seen independent of sex or age of the animals and

also across different strains. Even more remarkably, the effects are long lasting. After a recovery phase of four to eight weeks, still no elevation of neutrophil numbers was detected upon re-exposure to the trained stressor.

To investigate on which level of the stress response an adaption to a known stressor occurs, we analyzed stress parameters in stress trained animals. In regards of corticosterone, adrenaline and noradrenaline, the stress-trained group experienced the same level of stress as a group exposed to the stressor for the first time. Also, the heart rate was comparable in both groups, arguing that global sympathetic activity and cardiac output are not affected by stress training. To see if neutrophil pools might be depleted after repeated stress exposure, we analyzed the bone marrow neutrophil reservoir. No changes in neutrophil numbers in the bone marrow were detected, arguing that the neutrophil reserve is replenished with newly produced or returning mature neutrophils. Next, we tested if the stress training effect depends on corticosterone and found that the drastic drop in B cell numbers upon stress is blunted in absence of corticosterone, while the training effect on neutrophils is independently mediated.

As one of the major fear centers of the brain, the amygdala has been shown to play a key role in processing fearful input. Ablation of the amygdala region, however, did not change the effect of stress training on neutrophils. Taken together, these findings argue that stress trained animals are not less afraid, therefore making it less likely that the adaptation to the stressor happens at the input site. Thus, we tested if the effects are due to intrinsic changes to the neutrophils. We adoptively transferred GFP+ bone marrow cells of stress-trained or non-trained mice in to GFP- recipient mice, which were also stress trained or non-trained. Interestingly, we found retention of neutrophils in the bone marrow, and therefore low peripheral GFP+ neutrophil numbers, only in stress trained recipients. The effects were independent of the stress training status of the transplanted neutrophils. We conclude that the adaptation to stress is most likely happening in the bone marrow niche, which possibly prevents the release of neutrophils into the periphery upon re-exposure to trained psychological stressors. This effect could be mediated by upregulation of retention factor CXCL12 in bone marrow niche cells.

As we previously discussed, neutrophil release due to a psychological stressor can have detrimental effects on health. Since neutrophils may negatively affect the stability of atherosclerotic plaques, we analyzed aortas of stress-trained mice. These analyses revealed that in stress-trained mice the accumulation of neutrophils in plaques is completely blunted upon re-exposure to the trained stressor.

However, neutrophils have a crucial role in the defense against infection, injury and wound healing. In these incidences less neutrophil swarming would be detrimental. Our study shows that stress training induced retention of neutrophils in the bone marrow is overruled by infection and sterile liver injury, making the adaptation of the bone marrow niche in response to psychological stress not only effective, but highly sophisticated and flexible.

Summarizing, we showed that the immune response to psychological stress is a complex system. When talking about psychological stress, we need to distinguish between first time exposure or re-exposure to a trained stressor. An acute newly experienced stressor is met with a rapid, efficient but also metabolically costly response. Neutrophils are released from the bone marrow niche and swarm the periphery to build a first line of defense in case of injury. However, this comes at the risk of destabilizing atherosclerotic plaques and therefore myocardial infarction. Resilience to stress is a hot topic of our time. Other than the active and conscious adaptation to a stressor, we could show that upon stress training a form of adaptation happens on a cellular level. While changes in the bone marrow niche seem to retain neutrophils upon a re-exposure to a trained stressor, these effects do not overrule the release and therefore beneficial effects of neutrophil swarming in infection or injury.
On the first look, exposure to psychological stress does not seem desirable. Our research however clearly shows that it is more complicated. Our study unravels additional nuance to how acute stress affects leukocyte migration and function in different diseases and injury. For the first time, our study shows adaptation, likely of the bone marrow niche, upon re-exposure to a known stressor. These results might give us hints on how resilience to stress is mediated on a cellular level. Casually speaking, we could confirm, that in our setting of stress exposure, what does not kill you, indeed makes you stronger by cellular adaptation to the stressor.

Methods

Animals: Wild-type C57BL/6J, Apoetm1Unc (Apoe-/-), BALB/cJ and C57BL/6-Tg(UBC-GFP)30Scha/J (UBI-GFP) mice were purchased from The Jackson Laboratory, Maine, USA. Genotyping was performed as described on the website. Age- and sex-matched mice were used if not otherwise indicated at 8 to 16 weeks of age. Both male and female mice were used. All mice were group-housed before they were put into separate cages for single housing. Animals were randomly assigned to intervention groups. All procedures were approved by the Animal Review Committee at Massachusetts General Hospital (protocol no. 2011N000035 and 2015N000044) and were in compliance with relevant ethical regulations.

In vivo interventions

Diet: Apoe-/- mice were fed a high-fat diet (HFD, Harland Teklad) for 8-16 weeks prior to the experimental readouts. If not otherwise indicated, all other mice were fed a regular chow diet in compliance with animal housing regulations of the local branch of the Institutional Animal Care and Use Committee (IACUC) at Massachusetts General Hospital.

Stress: For stress studies, mice were single housed and allowed to habituate before any interventions were performed. Control mice were also housed in individual cages and treated alike apart from exposure to the stressor.
New cage stress and predator odor exposure: As a form of relatively mild acute stress, mice were placed into clean new cages for 1 hour before their blood leucocytes and corticosterone levels were analyzed. To study the stress response after exposure to predator odor small pieces of bobcat and fox urine-soaked cloths were placed into a corner of the cage for 1hrs.
Restraint stress: Mice were individually placed in restraint cylinders perforated with ventilation holes to ensure air exchange. This well-established procedure induces acute stress. We chose a procedure duration of 1-4h as indicated, because some of the effects of restraint stress only reach full expression after 4h (e.g. B cell migration to the bone marrow). The mice experience no pain or homeostatic challenges and are not physically squeezed. Mice were closely monitored while in the restraint device and transferred to their home cage after the procedure. Restraint is a very common and purely psychological stress procedure in mice. For stress training studies, mice were put into restrainers daily for the indicated number of episodes.

Intravital microscopy: Mice were restraint stressed for one hour, before they were anesthetized with 3% isoflurane and intravenously injected with a PE-labeled anti Ly6G antibody to visualize neutrophils (directly labelled fluorescent antibody against Ly6G in PE). Additionally, the fluorescent dye FITC dextran was used to label the vasculature. Mice were kept under

continuous anesthesia and the ear was placed under a custom-built confocal two-photon hybrid microscope for imaging.

Procedures: Peripheral sympathectomy was achieved by intraperitoneal injection of 6-OHDA (6-hydroxydopamine, 5mg per mouse) twice nine days apart. Saline injected mice served as controls.

Adrenalectomy: Mice were anesthetized with 5% isoflurane and upon unresponsiveness, isoflurane was reduced to 2-3% for continuous anesthesia. The body temperature was continuously maintained via a heating pad. After hair removal and disinfection, bilateral incisions were made cranial to the kidneys right below the ribcage. Adrenal glands were located and exposed. Without applying pressure, adrenal glands were removed with microsurgical forceps and scissors. Incisions were closed with two layers of sutures and after disinfection, mice were allowed to recover single housed on heating pads. For pain relief, mice were repetitively treated with Buprenorphine following the IACUC approved animal protocol.

Heart Rate telemetry: The telemetry device, composed of sensors, a battery and a transmitter, sends out physiological data through radio waves which are captured by a receiving device and forwarded to a computer for data storage and analysis. Implanted animals emit data remotely and allow data collection in a hands-off measurement period. For initial implantation surgery, mice were deeply anesthetized with 5% isoflurane and upon unresponsiveness (ensured by toe pinch), isoflurane was reduced to 2-3% for continuous anesthesia. The body temperature was continuously maintained via a heating pad. After hair removal and disinfection with 70% isopropanol, a skin incision was made from the thorax along the midline to the abdomen with microsurgical equipment. The probe was implanted into the right upper abdominal cavity. The abdominal cavity was closed with sutures. A thoracic midline skin incision was performed, to guide one electrode to the area of the sternocleidomastoid muscle and the other one to the fascia above the caudal sternum where they were fixed with sutures. Additionally, subcutaneous space was created for the electrodes. The skin was disinfected and closed with sutures. Mice were closely monitored until full recovery from anesthesia.

For pain relief, mice were repetitively treated with Buprenorphine following the IACUC approved animal protocol.

Amygdala ablation: Mice were deeply anesthetized with 5% isoflurane and upon unresponsiveness (ensured by toe pinch), isoflurane was reduced to 2-3% for continuous anesthesia. The head of the mouse was placed in a stereotactic frame by adjustment of ear

bars and with anesthesia continued via the mouse adapter. The body temperature was continuously maintained via a heating pad. After hair removal and disinfection with 70% isopropanol, an incision was made along the sagittal midline. Skull was exposed by removal of subcutaneous tissue. Horizontal placement of the skull was achieved by aligning the points Bregma and Lambda. After adjusting the stereotactic frame to amygdala coordinates, trepanation was achieved with a drill. The dura mater was mechanically opened and a prefilled Hamilton syringe was inserted according to depth coordinates. Over the time course of 3 minutes, 3x200 nl ibotenic acid (5% in PB) were injected per injection site (bilateral). Sham mice were prepared equally and injected with vehicle only. Surgical site was disinfected and the skin closed with sutures. Mice were allowed to recover from anesthesia single housed on a heating pad.
For pain relief, mice were repetitively treated with Buprenorphine following the IACUC approved animal protocol.

Surgical sponge implantation: Mice were either restrain stressed for 2 hours or remained relaxed, according to allocation of treatment groups. Immediately after the period of stress exposure, mice were anesthetized with isoflurane and a small skin incision was made on the back of the mouse, after hair removal and disinfection. Small sterile PBS-soaked surgical cellulose sponges were subcutaneously implanted and incisions were closed with sutures. The sponges were explanted, squeezed, and cut into small fragments to analyze their cellular content by flow cytometry 18 hours later.
For pain relief, mice were repetitively treated with Buprenorphine following the IACUC approved animal protocol.

Bone marrow transplantation: Male GFP-Ubi donor mice, 8-16 weeks old, were sacrificed and bone marrow cells were collected from femur, tibia, scapula and pelvic bones. After red blood cell lysis, cell numbers were determined using counting beads and flow cytometry. Recipient mice were anesthetized with 5% isoflurane and intravenously injected with approximately $18x10^6$ bone marrow cells. Mice were allowed to recover for 10 hours before further manipulation.

LPS infection model: A group of relaxed naïve mice and relaxes stress-trained mice were injected i.p. with 100 µg/kg LPS. Blood was collected 1 and 5 hours after injection and mice were sacrificed 24 hours after LPS injection.

Sterile liver injury model: A group of relaxed naïve mice and relaxes stress-trained mice were deeply anesthetized with 5% isoflurane and upon unresponsiveness, isoflurane was reduced

to 2-3% for continuous anesthesia. After hair removal and disinfection, an abdominal incision was made. The peritoneum was opened to expose a small segment of liver tissue. Sterile liver injury was induced by briefly advancing a hot surgical cauter into the liver tissue. This intervention resulted in a round thermal injury site of approx. 2mm in diameter. After closing the surgical site with sutures, mice were allowed to fully recover single housed on a heating pad.
For pain relief, mice were repetitively treated with Buprenorphine following the IACUC approved animal protocol.

Cells

Cell collection: Peripheral blood was collected by bleeding and lysed in RBC lysis buffer (Biolegend). After PBS perfusion, spleens were crushed through a 40 µm cell strainer and lysed. Bone marrow single cells were collected by flushing bones with PBS, pipetting up and down, and lysis of red blood cells. Other organs (liver, lung, skin, muscle, aorta) were collected and after mincing with scissors digested with collagenase I, collagenase XI, DNase I and hyaluronidase in PBS for 30 mins to 1 h at 37°C.

Flow cytometry: To remove any remaining red blood cells, cells were treated with red blood cell lysis buffer for 5-10 mins at room temperature. Single cell suspensions were stained in buffer containing 2mM EDTA and 0.5% BSA. The following monoclonal antibodies were used for flow cytometry analyzes: Ly6C, Ly6G, CD11b, CD3, CD90.2, CD19, CD4, CD8a, NK1.1, CD45, CX3CR1, MHCII, CD62L; Single cell suspensions were stained with antibody mix for 15 mins at 4° Celsius. Thereafter, a fixable viability dye was used to differentiate living from dead/apoptotic cells. Cells were either analyzed fresh or fixed with 150µl of Cytofix (BD Biosciences, California) for 15-20 mins and subsequently analyzed. Precision count beads (Biolegend) were added in order to determine absolute cell numbers. Data from stained single cell suspensions was acquired on a LSRII (BD Bioscience) and analyzed with FlowJo software (Treestar inc.).

Biochemistry assays

Enzyme-linked immunosorbent assay (ELISA): Plasma stress hormone levels were measured using ELISA kits according to the manufacturers' instructions. The Corticosterone ELISA kit from Abcam was used to detect and quantify corticosterone content in plasma samples (previously stored at -80° Celsius) via a competitive colorimetric detection method.
For ultrasensitive determination of catecholamines (adrenalineand noradrenaline), a 2-CAT research ELISA by LDN was used on previously frozen plasma samples.

Histology

Perfusion Fixation: Mice were deeply anesthetized with 5% isoflurane and fixated in supine position. Skin and peritoneum were opened with an incision. The thorax was opened by parasternal incision and the heart was carefully freed from fat tissue. The right auriculum of the heart was opened to allow unhindered perfusion. A cannula was inserted into the left ventricle and animals were perfused with 10ml of PBS followed by 10ml of paraformaldehyde to fix brain or aortic tissue. Immediately after perfusion, the skull was opened and brains removed. Brains were kept in paraformaldehyde for 24hrs before transferring them into 0.8M sucrose for cryoprotection.

Fixated brains were cut into predefined coronal blocks. The blocks and aortic roots were embedded in O.C.T. Tissue Tek Compound from Sakura. Samples were flash frozen in 2-Methylbutan cooled with dry ice. Frozen samples were kept at -80° Celsius until sectioning.

Cryosectioning: Coronal brain sections (50µm) and aortic roots (5µm) were sectioned on a cryomicrotome. Sections were collected in PBS prefilled glasses to produce series of sections with a distance of 200µm between two sections of one series. If not immediately stained, sections were transferred and stored at -20° Celsius in an anti-freeze solution, containing sucrose and ethylene glycol.

Tissue staining: Free floating 50µm brain or aortic root sections were pre-washed in PBS to remove excess of O.C.T. embedding media. Sections were pre-treated with 1% sodium borohydride in PBS for 15 minutes and washed 3-5 times with PBS. This step ensures to remove excessive aldehydes. Preincubation and blocking of unspecific binding sites was done in 10% normal horse serum, 0.3% Triton-X and 0.05% phenylhydrazine in PBS for 30 minutes. This mixture additionally increases the membrane permeability of the tissue, as well as it blocks endogenous peroxidase activity. Sections were transferred in solutions of 10% normal horse serum, 0.3% Triton-X and 0.1% sodium azide mixed with the respective primary antibody in previously determined dilutions for 24 hours at 4° Celsius. After two washing steps in PBS (20 mins and 40 mins), sections were preincubated with PBS-A for 60 mins and then transferred to PBS-A solution containing the secondary antibody, as well as 0.3% Triton-X and 0.1% sodium azide for 2 to 4 hours at room temperature.

Immunofluorescent staining: Aortic root cryo sections were immunofluorescent stained against DAPI and with directly labeled secondary antibodies Ly6G. Sections were mounted on gelatin-coated slides, air dried and coverslipped with Vectashield antifade mounting medium for fluorescence.

NeuN staining: To visualize neurons, NeuN staining was performed with a rabbit anti NeuN primary antibody and subsequently a biotin labelled anti-rabbit secondary antibody was used as described above. Thereafter, sections were washed with PBS, preincubated with PBS-A for 60 minutes, and incubated with avidin-biotin-elite complex (ABC, diluted 1:200) in PBS-A for 2 hours. The ABC's streptavidin component forms a complex with the biotinylated secondary antibody and a peroxidase attaches to free binding sites of the resulting antibody-complex. After another washing step, free floating sections were incubated in solution containing 3.3-diaminobenzidine (DAB) and imidazole in a Tris buffer for 15 mins. The reaction was developed by adding 0.03% hydrogen peroxide to the DAB solution and stopped after 10 to 15 minutes with PBS.

Brain sections were mounted on gelatin coated slides and air dried. The tissue was dehydrated via a graded series of ethanol (70%, 80%, 90% and 100%) and transferred shortly into xylene. In a final step Entellan was used as coverslip mounting medium.

Literature

[1] Workplace Stress Management Market Size | Industry Report, 2026 n.d. https://www.grandviewresearch.com/industry-analysis/workplace-stress-management-market/toc (accessed July 11, 2020).
[2] Stress: Signs, Symptoms, Management & Prevention n.d. https://my.clevelandclinic.org/health/articles/11874-stress (accessed July 11, 2020).
[3] Cannon WB. Bodily changes in pain, hunger, fear and rage: An account of recent researches into the function of emotional excitement. D Appleton & Company; 1915. doi:10.1037/10013-000.
[4] Nature HS-, 1936 undefined. A syndrome produced by diverse nocuous agents. NatureCom n.d.
[5] Inc. HS-, Montreal undefined, Canada undefined, 1950 undefined. The physiology and pathology of exposure to stress. ACTA n.d.
[6] Selye H. Stress without Distress BT - Psychopathology of Human Adaptation. Serban G (Eds) Psychopathol Hum Adapt 1976:137–46. doi:10.1007/978-1-4684-2238-2_9.
[7] Bienertova-Vasku J, Lenart P, Scheringer M. Eustress and Distress: Neither Good Nor Bad, but Rather the Same? BioEssays 2020;42:1900238. doi:10.1002/bies.201900238.
[8] Ronald De Kloet E, Joels M. Stress and the brain: from adaptation to disease. NatureCom 2005;6:463–75. doi:10.1038/nrn1683.
[9] Janak PH, Tye KM. From circuits to behaviour in the amygdala. Nature 2015;517:284–92. doi:10.1038/nature14188.
[10] Cramer GD, Darby SA. Clinical Anatomy of the Spine, Spinal Cord, and ANS. 2013. doi:10.1016/C2009-0-42801-0.
[11] Joëls M, Baram TZ. The neuro-symphony of stress. Nat Rev Neurosci 2009;10:459–66. doi:10.1038/nrn2632.
[12] William Tank A, Lee Wong D. Peripheral and Central Effects of Circulating Catecholamines. Compr. Physiol., vol. 5, Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2014, p. 1–15. doi:10.1002/cphy.c140007.
[13] Boulpaep E, Boron W, Caplan M, Transduct LC-S, 2009 undefined. Medical Physiology : A Cellular and Molecular approach. n.d.
[14] Vale W, Rivier C, Yang L, Minick S, Guillemin R. Effects of Purified Hypothalamic Corticotropin-Releasing Factor and Other Substances on the Secretion of Adrenocorticotropin and β-Endorphin-Like Immunoactivities in Vitro. Endocrinology 1978;103:1910–5. doi:10.1210/endo-103-5-1910.
[15] Herman JP, Figueiredo H, Mueller NK, Ulrich-Lai Y, Ostrander MM, Choi DC, et al. Central mechanisms of stress integration: Hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. Front Neuroendocrinol 2003;24:151–80. doi:10.1016/j.yfrne.2003.07.001.
[16] Liu JJ, Buisman-Pijlman F, Hutchinson MR. Toll-like receptor 4: Innate immune regulator of neuroimmune and neuroendocrine interactions in stress and major depressive disorder. Front Neurosci 2014;8. doi:10.3389/fnins.2014.00309.
[17] Wohleb ES, McKim DB, Sheridan JF, Godbout JP. Monocyte trafficking to the brain with stress and inflammation: A novel axis of immune-to-brain communication that influences mood and behavior. Front Neurosci 2015;9. doi:10.3389/fnins.2014.00447.
[18] Powell ND, Sloan EK, Bailey MT, Arevalo JMG, Miller GE, Chen E, et al. Social stress up-regulates inflammatory gene expression in the leukocyte transcriptome via β-adrenergic induction of myelopoiesis. Proc Natl Acad Sci U S A 2013;110:16574–9. doi:10.1073/pnas.1310655110.
[19] Heidt T, Sager HB, Courties G, Dutta P, Iwamoto Y, Zaltsman A, et al. Chronic variable stress activates hematopoietic stem cells. Nat Med 2014;20:754–8. doi:10.1038/nm.3589.
[20] Swirski FK, Nahrendorf M. Leukocyte Behavior in Atherosclerosis, Myocardial Infarction, and Heart Failure. Science (80-) 2013;339:161–6. doi:10.1126/science.1230719.
[21] Burns VE, Ring C, Drayson M, Carroll D. Cortisol and cardiovascular reactions to mental stress and antibody status following hepatitis b vaccination: A preliminary study. Psychophysiology 2002;39:361–8. doi:10.1017/S0048577201393022.
[22] Burns VE, Drayson M, Ring C, Carroll D. Perceived stress and psychological well-being are associated with antibody status after meningitis C conjugate vaccination. Psychosom Med 2002;64:963–70. doi:10.1097/01.PSY.0000038936.67401.28.
[23] Burns VE, Carroll D, Ring C, Harrison LK, Drayson M. Stress, coping, and hepatitis B antibody

status. Psychosom Med 2002;64:287–93. doi:10.1097/00006842-200203000-00012.
[24] Cohen S, Miller GE, Rabin BS. Psychological stress and antibody response to immunization: A critical review of the human literature. Psychosom Med 2001;63:7–18. doi:10.1097/00006842-200101000-00002.
[25] Glaser R, Sheridan J, Malarkey WB, MacCallum RC, Kiecolt-Glaser JK. Chronic stress modulates the immune response to a pneumococcal pneumoaia vaccine. Psychosom Med 2000;62:804–7. doi:10.1097/00006842-200011000-00010.
[26] Marsland AL, Cohen S, Rabin BS, Manuck SB. Associations between stress, trait negative affect, acute immune reactivity, and antibody response to hepatitis B injection in healthy young adults. Heal Psychol 2001;20:4–11. doi:10.1037/0278-6133.20.1.4.
[27] McGregor BA, Murphy KM, Albano DL, Ceballos RM. Stress, cortisol, and B lymphocytes: A novel approach to understanding academic stress and immune function. Stress 2016;19:185–91. doi:10.3109/10253890.2015.1127913.
[28] Igarashi H, Medina KL, Yokota T, Rossi MID, Sakaguchi N, Comp PC, et al. Early lymphoid progenitors in mouse and man are highly sensitive to glucocorticoids n.d. doi:10.1093/intimm/dxh230.
[29] Lill-Elghanian D, Schwartz K, King L, Fraker P. Glucocorticoid-induced apoptosis in early B cells from human bone marrow. Exp Biol Med 2002;227:763–70. doi:10.1177/153537020222700907.
[30] De Kloet ER, Sibug RM, Helmerhorst FM, Schmidt M. Stress, genes and the mechanism of programming the brain for later life. Neurosci. Biobehav. Rev., vol. 29, Elsevier Ltd; 2005, p. 271–81. doi:10.1016/j.neubiorev.2004.10.008.
[31] Naeim F, Nagesh Rao P, Song SX, Phan RT. Granulocytic Disorders. Atlas Hematop., Elsevier; 2018, p. 871–84. doi:10.1016/b978-0-12-809843-1.00063-2.
[32] Furze RC, Rankin SM. Neutrophil mobilization and clearance in the bone marrow. Immunology 2008;125:281–8. doi:10.1111/j.1365-2567.2008.02950.x.
[33] Patchev VK, Patchev A V. Experimental models of stress. Dialogues Clin Neurosci 2006;8:417–32.
[34] Koolhaas JM, de Boer SF, Buwalda B, Meerlo P. Social stress models in rodents: Towards enhanced validity. Neurobiol Stress 2017;6:104–12. doi:10.1016/j.ynstr.2016.09.003.
[35] Kaplan JR, Manuck SB, Clarkson TB, Lusso FM, Taub DM, Miller EW. Social stress and atherosclerosis in normocholesterolemic monkeys. Science (80-) 1983;220:733–5. doi:10.1126/science.6836311.
[36] Gu H, Tang C, Peng K, Sun H, Yang Y. Effects of Chronic Mild Stress on the Development of Atherosclerosis and Expression of Toll-Like Receptor 4 Signaling Pathway in Adolescent Apolipoprotein E Knockout Mice. J Biomed Biotechnol 2009:13. doi:10.1155/2009/613879.
[37] Bernberg E, Ulleryd MA, Johansson ME, Bergström GML. Social disruption stress increases IL-6 levels and accelerates atherosclerosis in ApoE -/- mice. Atherosclerosis 2012;221:359–65. doi:10.1016/j.atherosclerosis.2011.11.041.
[38] Wilbert-Lampen U, Leistner D, Greven S, Pohl T, Sper S, Völker C, et al. Cardiovascular Events during World Cup Soccer. N Engl J Med 2008;358:475–83. doi:10.1056/NEJMoa0707427.
[39] Cesarovic N, Jirkof P, Rettich A, Arras M. Implantation of radiotelemetry transmitters yielding data on ECG, heart rate, core body temperature and activity in free-moving laboratory mice. J Vis Exp 2011;57:3260. doi:10.3791/3260.