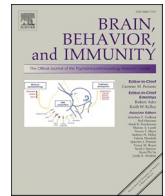


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Short Communication

Acute stress increases monocyte levels and modulates receptor expression in healthy females

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ABSTRACT

There has been a growing recognition of the involvement of the immune system in stress-related disorders. Acute stress leads to the activation of neuroendocrine systems, which in turn orchestrate a large-scale redistribution of innate immune cells, such as monocytes. Even though acute stress/monocyte interactions have been well-characterized in mice, this is not the case for humans. As such, this study aimed to investigate whether acute stress modulates blood monocyte levels in a subtype-dependent manner and whether the receptor expression of stress-related receptors is affected in humans. Blood was collected from healthy female volunteers at baseline and 1 h after the socially evaluated cold pressor test, after which blood monocyte levels and receptor expression were assessed by flow cytometry. Our results reveal a stress-induced increase in blood monocyte levels, which was independent of monocyte subtypes. Furthermore, colony stimulating factor 1 receptor (CSF-1R) and CD29 receptor expression was increased, while CD62L showed a trend towards increased expression. These results provide novel insights into how acute stress affects the innate immune system.

1. Introduction

Stress-related psychopathologies, such as anxiety and depression, are the most prevalent mental health disorders worldwide and impose a significant burden on society (WHO, 2017). These psychopathologies have repeatedly been linked to the immune system (Dantzer et al., 2008; Langgartner et al., 2019; Wohleb et al., 2016). In particular activation of the innate immune system has received significant attention, as patients with major depressive disorder have been reported to have an enhanced pro-inflammatory monocyte profile (Nowak et al., 2019), while chronic stress increases monocyte levels in humans (Heidt et al., 2014). Chronic stress has been shown to increase monocyte trafficking, which is associated with neuroinflammation and deficits in anxiety-like behaviour and anhedonia in male mice (Wohleb et al., 2013; Mackos et al., 2016; Zheng et al., 2016; Gururajan et al., 2019), and female mice (Yin et al., 2019). Similar to chronic stress, acute stress changes circulating innate immune cell levels in mice (Boehme et al., 2020; Dhabhar et al., 1994,

2012; van de Wouw et al., 2019b), where immune cells migrate into tissues such as the skin (Dhabhar and McEwen, 1996). As such, understanding how acute stress affects monocytes will provide insight into the development of chronic stress and stress-related disorders.

It has recently been reported that acute stress reduces circulating monocyte levels in a subtype-dependent level in mice (van de Wouw et al., 2019b). The subtype most affected by acute stress is the LY6C^{hi} monocyte subtype, which has been linked to inflammatory responses (Jakubzick et al., 2017; Williams et al., 2018). Even though acute stress decreases blood monocyte levels in humans (Brazaitis et al., 2014), it is still unclear whether this is in a subtype-dependent manner.

The increase in glucocorticoid signalling induced by acute stress has been shown to mediate immune cell trafficking in mice and humans (Dhabhar et al., 2012; Olnes et al., 2016; Yeager et al., 2016). Conversely, this increase in glucocorticoid levels in response to acute stress has been associated with an anti-inflammatory effect (Miller et al., 2002; Kunz-Ebrecht et al., 2003). Indeed, incubation of human and

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murine monocytes with glucocorticoids *ex vivo* induces an anti-inflammatory phenotype (Ehrchen et al., 2007; Varga et al., 2008, 2014; Tsianakas et al., 2012). This may indicate that, even though acute stress results in a systemic immune activation (Marsland et al., 2017), monocytes may be affected by acute stress in an anti-inflammatory manner on a cellular level through glucocorticoid signalling. This is especially important because repeated acute stressors (i.e. chronic stress) induce monocyte trafficking (Reader et al., 2015), which has been associated with neuroinflammation (Wohleb et al., 2013; Zheng et al., 2016). This indicates the importance of understanding how acute stress modulates monocytes on a cellular level.

As such, the objectives of this study were twofold: 1) Does acute stress modulate human monocyte levels in a subtype-dependent manner? 2) Does acute stress modulate human monocytes on a cellular level as measured by receptor expression? Aim 1 was investigated by comparing blood monocyte subtype levels (i.e. classical, intermediate, and non-classical monocytes) at baseline and after acute stress using flow cytometry. Aim 2 was assessed by quantifying receptor expression of receptors previously reported to be changed by glucocorticoids, or acute or chronic stress. These receptors include the colony stimulating factor 1 receptor (CSF-1R) (Wohleb et al., 2018), innate immune system activation markers (CD163 and CD14) (Ehrchen et al., 2007), trafficking receptors (CCR2 and CX3CR1) (Okutsu et al., 2008; Prinz and Priller, 2010; Yeager et al., 2016) and adhesion receptors (CD29, CD11b and CD62L) (Dhabhar et al., 2012; Sawicki et al., 2015).

2. Methodology

2.1. Subjects

The clinical research described was part of a larger study and received approval from the Clinical Research Ethics Committee of the Cork Teaching Hospitals (Protocol Number: APC076). The study was conducted under the ICH Guidelines on Good Clinical Practice. Written informed consent was obtained from all participants before any study procedures were performed, and participants were free to withdraw from the study at any time.

These data were derived from participants from a larger healthy volunteer study examining the effects of fiber intervention on the microbiota-gut-brain-axis, which also contains population characteristics (Berdig et al., 2020). No effect of fiber intervention was observed on any of the reported findings in this manuscript. Briefly, the following inclusion criteria were used: able to give written informed consent; female; between 18 and 40 years of age; in generally good health as determined by the investigator. The exclusion criteria were: have a significant acute or chronic coexisting illness or any condition which contraindicates, in the investigators' judgement, entry to the study; have a condition or taking a medication such as anxiolytics, antipsychotics, antidepressants, anticonvulsants, centrally acting corticosteroids, opioid pain relievers, laxatives, enemas, antibiotics, anti-coagulants, and over-the-counter non-steroidal anti-inflammatories (NSAIDs); have used pre- or probiotics over the last 4 weeks; are peri-menopausal, menopausal or post-menopausal; are pregnant or planning a pregnancy, or lactating; are vegan; are a current or past habitual daily smoker; individuals who, in the opinion of the investigator, are considered to be poor attendees or unlikely for any reason to be able to comply with the trial; subjects receiving treatment involving experimental drugs; have a malignant disease or any concomitant end-stage organ disease.

2.2. Socially evaluated cold pressor test

The socially evaluated cold pressor test (SECPT) combining a psychological with a physiological stressor was utilized to elicit an acute stress response as previously described (Schwabe et al., 2008; Allen et al., 2016). Briefly, the participant was led into the room where the SECPT would take place, where a researcher was present with a video

camera. The participant was told that their facial expressions would be video recorded for later analysis and that the researcher was specially trained to monitor their non-verbal behavior. They were then asked to submerge their hand in water containing ice (i.e., 0 °C) for three minutes unless the participant indicated they could no longer continue. After these three minutes, the participant was given a paper towel to dry their hand and allowed to leave the room.

2.3. Human PBMC isolation

Blood was collected in 4 mL lithium-heparin containing tubes (Greiner Bio-One, 454029) before, and 1 h after the SECPT. Blood tubes were centrifuged at 1500g for 10 min at 4 °C, after which plasma was collected. The collected volume of plasma was replaced with RPMI-1640 medium with L-glutamine and sodium bicarbonate (Sigma-Aldrich, R8758) and blood samples were further diluted with medium (1:1 dilution). Samples were carefully layered on 4.5 mL Ficoll® Paque Plus (Sigma-Aldrich, GE17-1440-02). Tubes were centrifuged at 450g for 30 min at 4 °C without breaks. Mononuclear cells were collected, washed with 5 mL medium and centrifuged at 300g for 10 min at 4 °C. Supernatant was aspirated and cells were resuspended in 1 mL medium. Cell numbers were counted with an automatic cell counter (Countess, Invitrogen), after which 2×10^6 cells were used for flow cytometry.

2.4. Flow cytometric analysis

Cells were centrifuged at 1500g for 5 min at 4 °C, washed with 1 mL phosphate-buffered saline, and centrifuged once more. Pellets were resuspended in 500 μ L BD Horizon™ Fixable Viability Stain 780 (BD Biosciences, 565388) and incubated for 15 min at room temperature. 1 mL Miltenyi buffer (autoMACS Rinsing Solution (Miltenyi, 130-091-222) supplemented with MACS BSA stock solution (Miltenyi, 130-091-376)) was added to the samples and centrifuged. Cell pellets were subsequently resuspended in 50 μ L Brilliant Stain Buffer (BD Biosciences, 563794), after which 5 μ L FcR blocking reagent (Miltenyi Biotec, 130-059-901) was added. Cell suspensions were incubated for 10 min on ice and the mix of antibodies was added (sTable 1). After a 30-minute incubation on ice, 1 mL Miltenyi buffer was added, samples were centrifuged, and cell pellets were resuspended in 4% paraformaldehyde for 30 min. Finally, 1 mL Miltenyi buffer was added, samples were centrifuged, and cell pellets were resuspended in Miltenyi buffer for flow cytometric analysis the following day on the BD FACSCelesta (BD Biosciences). Data were analysed using FlowJo (version 10). Gating of monocyte subtypes was performed as previously discussed (Villani et al., 2017; Berdig et al., 2020). Briefly, cells were first selected based on FSC/SSC, after which doublets were excluded. Live cells (FVS780-) were selected, after which SSC^{high} cells (granulocytes) and DUMP- cells (CD3+ T cells, CD19+ B cells, and CD56+ NK cells) were excluded. Monocyte subtypes were subsequently selected based on CD14 and CD16 receptor expression. Cell numbers were normalized to total live single-cell numbers. Receptor expression was assessed using the median fluorescent intensity (MFI).

2.5. Statistical analysis

Data were non-parametrically distributed. Differences between baseline and stress were analysed using the non-parametric paired Wilcoxon signed-rank test. SPSS version 26 was used for the statistical analysis. Data are depicted as box plots showing the median and quartiles with individual data points. Error bars depict the min and max. $P < 0.05$ was deemed significant.

3. Results

3.1. Acute stress mobilizes peripheral monocytes

Acute stress increased the levels of all monocyte subtypes (classical monocyte: $Z = -2.215$, $p = 0.027$; intermediate monocytes: $Z = -2.556$, $p = 0.011$; non-classical monocytes: $Z = -3.408$, $p < 0.001$) (Fig. 1 and sFig. 1A). The relative stress-induced changes in monocyte levels were similar between the different monocyte subtypes (sFig. 1B). However, the change in cell number was markedly higher in classical monocytes, which is likely attributed to the increased prevalence of classical monocytes overall (sFig. 1C). We, therefore, primarily focussed on classical monocytes in further analyses.

3.2. Acute stress modulates monocyte receptor expression

Acute stress increased CSF-1R and CD29 receptor expression on classical monocytes ($Z = -2.981$, $p = 0.003$; $Z = -2.062$, $p = 0.039$, respectively) (Fig. 2A, C). In addition, acute stress induced a trend towards increased CD62L receptor expression on classical monocytes ($Z = -1.874$, $p = 0.061$) (Fig. 2B). No differences were observed in CD11b, CD163, CD14, CCR2 and CX3CR1 receptor expression on classical monocytes (Fig. 2D–H). Similar to classical monocytes, acute stress increased CSF-1R and CD62L receptor expression in intermediate and

non-classical monocytes (sFig. 2A–D). Acute stress also induced a trend towards decreased CD14 expression in intermediate monocytes, but not non-classical monocytes (sFig. 2E, F). No other significant changes in receptor expression were observed in intermediate and non-classical monocytes.

3.3. Acute stress-induced changes in monocyte levels do not correlate with changes in receptor expression

There has been an increased emphasis on interindividual differences to acute stressor responses (i.e., responders vs non-responders) (Nielsen et al., 2013). As such, we correlated acute stress-induced changes in monocyte subtype levels with changes in receptor expression, as these should correlate with each other if individuals with large changes in classical monocytes would also have large changes in other monocyte subsets or receptor expression (sFig. 3). We did not find any clear evidence for responders vs non-responders to acute stress-induced changes in monocyte levels and receptor expression, as there was only a significant correlation between changes in intermediate monocytes with changes in non-classical monocytes ($p = 0.010$, 95% CI: [0.142, 0.991]), while all other parameters were not significantly different.

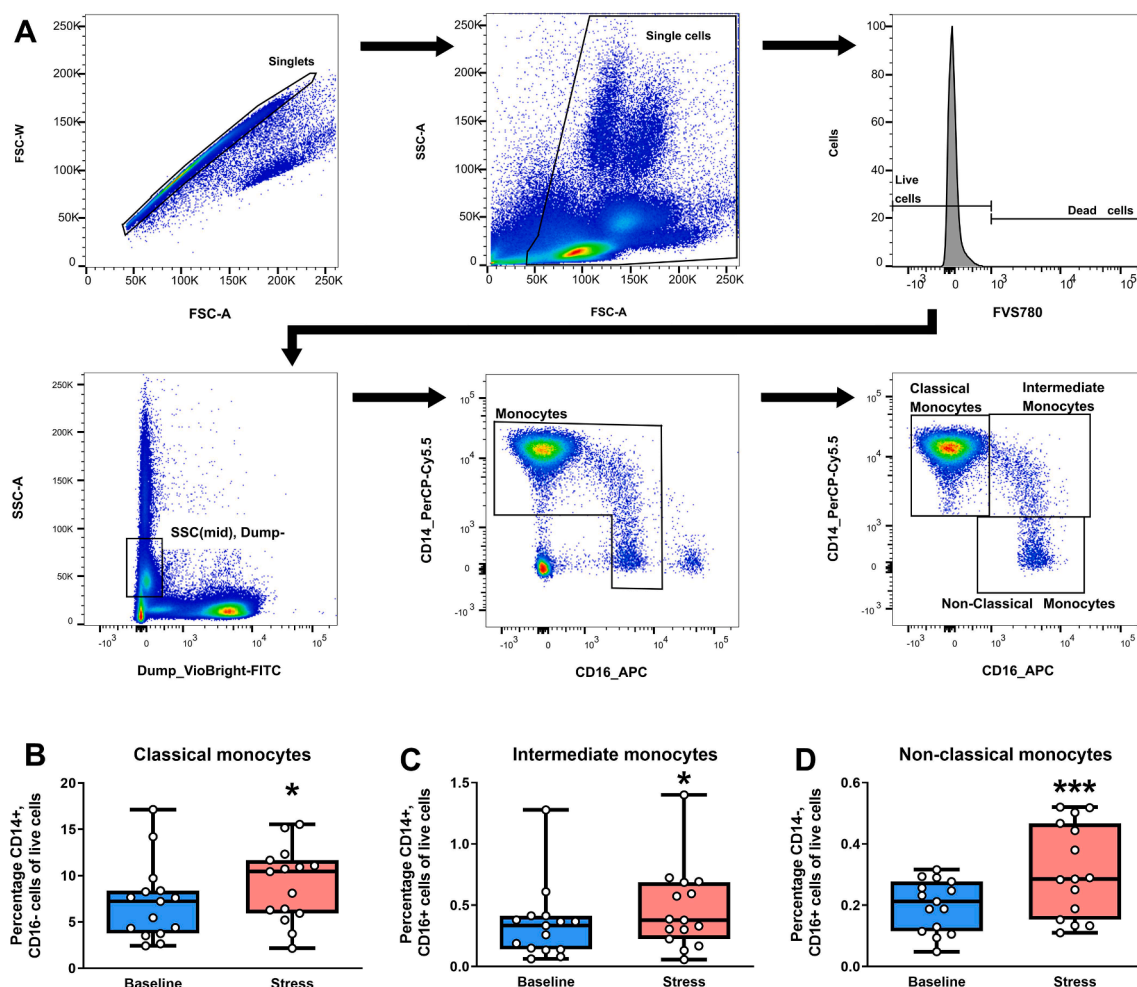


Fig. 1. Acute stress increased circulating monocyte levels. A) Monocytes were identified by first selecting singlets, after which single cells and subsequently live cells were gated. SSC^{mid} and DUMP⁻ cells were subsequently selected, after which monocytes were gated based on CD14 and CD16 receptor expression. Finally, monocyte subtypes were selected as classical monocytes (CD14⁺, CD16⁻), intermediate monocytes (CD14⁺, CD16⁺), and non-classical monocytes (CD14⁻, CD16⁺). B–D) Acute stress-induced changes were subsequently assessed in all monocyte subtypes using the non-parametric Wilcoxon signed-rank test. Statistical significance is depicted as * $P < 0.05$ and *** $P < 0.001$. Data are depicted as box plots with individual datapoints ($n = 15$).

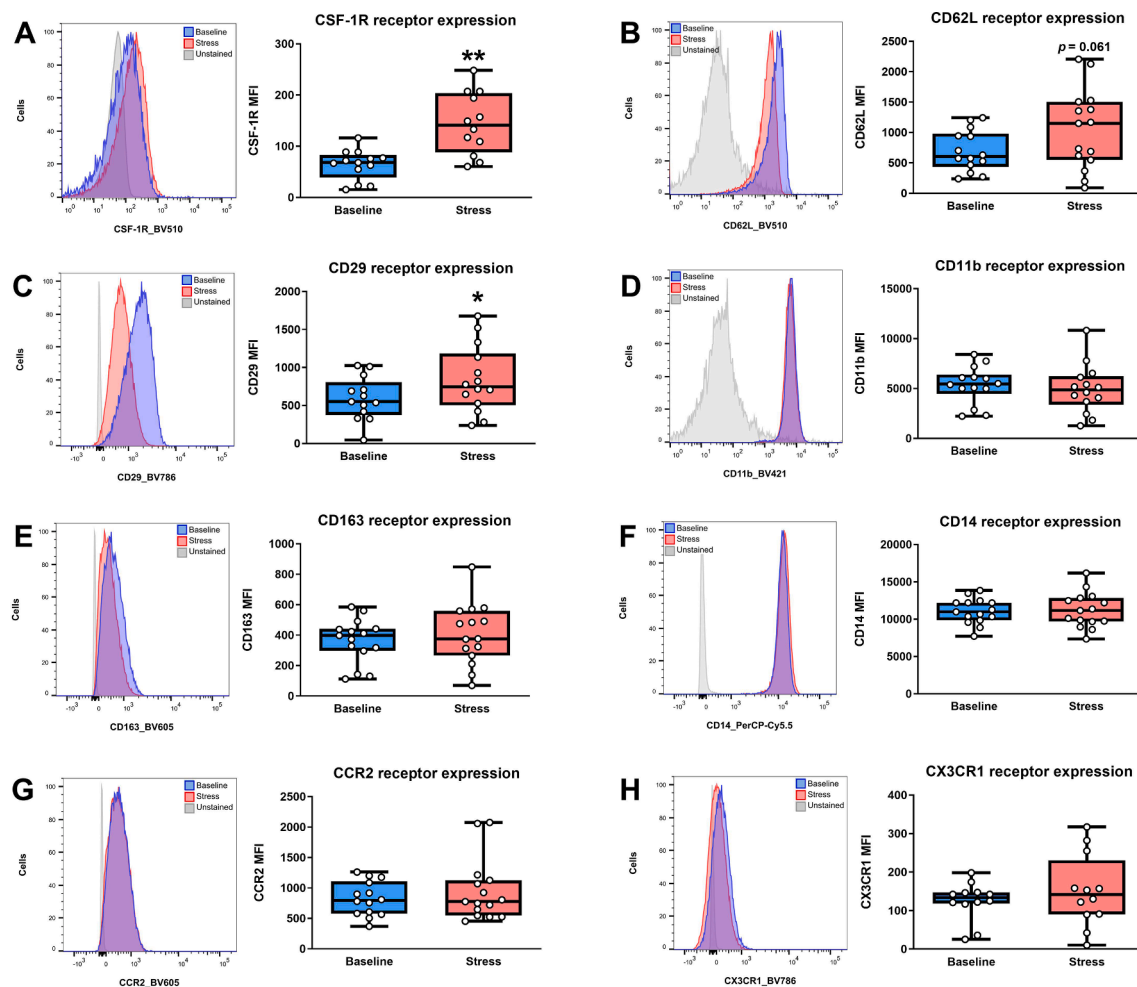


Fig. 2. Acute stress increased CSF-1R, CD29 and induced a trend towards increased CD62L receptor expression in classical monocytes. A-H) Acute stress-induced changes in CSF-1R, CD62L, CD29, CD11b, CD163, CD14, CCR2 and CX3CR1 receptor expression in classical monocytes was measured by median fluorescent intensity (MFI) and assessed using the non-parametric Wilcoxon signed-rank test. Statistical significance is depicted as * $P < 0.05$ and ** $P < 0.01$. Receptor staining plots are depicted on the left. Data are depicted as box plots with individual datapoints on the right ($n = 15$).

4. Discussion

This study demonstrates that an acute stressor increases circulating monocyte levels and CSF-1R and CD29 receptor expression on classical monocytes in healthy female individuals. In addition, there was a trend towards increased CD62L receptor expression.

In rodents, it has been repeatedly shown that acute stress decreases circulating monocyte levels (Dhabhar et al., 1994, 2012; van de Wouw et al., 2019b). Our data reveal that acute stress increases monocyte levels, which is the opposite of what has been reported in another study (Brazaitis et al., 2014). The discrepancy between mice and humans may be explained by the severity or nature of the stressor (i.e. restraint stress in rodents compared to the SECPT in humans in this study) or potentially sex. For instance, the SECPT stress in this study has a psychosocial component, where participants were told that they were monitored and videotaped, which is absent during restraint stress in rodents and was not done by Brazaitis et al. Indeed, different types of stress affect physiology and behavior in different ways in rodents (Du Preez et al., 2020). Alternatively, sex-specific differences in innate immune responses have been reported (Kovats 2015, Shepherd et al., 2020). For instance, *ex vivo* inflammatory responses of monocytes to multiple microbial stimuli are affected by sex (Ter Horst et al., 2016). Importantly, previous studies on acute stress-induced changes in monocyte levels have primarily been in male rodents and humans (Brazaitis et al., 2014; Dhabhar et al., 2012; van de Wouw et al., 2019b). The majority of

studies investigating the impact of chronic stress on monocyte trafficking and behavior have also been in male mice, even though some of these findings have been replicated in female mice (Wohleb et al., 2018; Yin et al., 2019). Specific stress-induced effects are also influenced by sex, as male mice show increased microglia activation and reduced dendritic spine density in the medial prefrontal cortex following chronic unpredictable stress compared to female mice (Wohleb et al., 2018). These results further highlight the need for the investigation into sex-specific effects of acute and chronic stress. Furthermore, one limitation to our study is not controlling for menstrual cycle or contraceptive use, which will also be crucial to assess in future studies.

A stress-induced increase in monocyte levels in humans could indicate that more monocytes are released from their reservoir than traffic into tissues. Acute stress has also been shown to reduce the LY6C^{hi} monocyte levels in the splenic reservoir in mice, indicating that acute stress may indeed recruit monocyte levels from their reservoir (van de Wouw et al., 2019b). The discrepancy between our increase in monocyte levels and the previously reported decrease may also be due to differences in techniques as the aforementioned work used an automated haematology analyser (Brazaitis et al., 2014). Automated haematology analysers have been reported to be less accurate compared to flow cytometry, especially for less abundant cell types such as monocytes (Buoro et al., 2018), which may indicate that the decrease in monocytes in this study may be reflective of decreases of other cell types. As such, more studies are warranted comparing different types of stressors and

immune panels containing more different types of immune cells.

Acute stress-induced changes in monocyte levels have also been shown to be LY6C^{hi} and LY6C^{mid}, CCR2⁺ monocyte subtype-specific in mice (van de Wouw et al., 2019b). This subtype has been linked to classical monocytes in humans (Jakubczick et al., 2017; Guillems et al., 2018). Our data reveal that acute stress-induced changes in human monocyte levels are not specific to subtypes. This might be explained by differences between mice and humans, as others have previously reported species-specific differences in monocytes (Shay et al., 2013; Reynolds and Haniffa, 2015).

Acute stress increased CSF-1R and CD29 receptor expression on classical monocytes and induced a trend towards increased CD62L receptor expression. Increases in colony stimulating factor-1, the ligand for CSF-1R, have been observed in male mice undergoing chronic unpredictable stress, which was linked to microglia-mediated neuronal remodeling and deficits in anxiety- and depressive-like behavior (Wohleb et al., 2018). Interestingly, CSF-1R has been implicated in monocyte differentiation into macrophages (Rojo et al., 2019), and it could be that acute stress-induced increases in CSF-1R expression may shift monocytes away from a pro-inflammatory state once the stress has ceased (Hume et al., 2019). Both CD29 and CD62L have been implicated in cell adhesion, indicating that acute stress may increase monocyte adhesion and migration (Ivetic et al., 2019). This may also facilitate monocytes to traffic into sites of inflammation, as for instance, acute stress increases leukocyte infiltration into inflamed skin of rats (Dhabhar and McEwen, 1996). It is interesting to note that chronic social defeat stress increases the expression of key adhesion molecules (Sawicki et al., 2015). Changes in CD62L receptor expression have been reported in rats exposed to acute stress (Dhabhar et al., 2012), while VCAM-1, the ligand for CD29, was upregulated in the murine brain in response to chronic defeat stress (Sawicki et al., 2015). As such, these findings may be relevant to stress-related disorders.

Overall, the results presented in this manuscript indicate that acute stress induces an inflammatory phenotype in monocytes at a receptor expression level in humans. This is in line with other findings showing an increase in circulating inflammatory cytokines in response to acute stress (Marsland et al., 2017). Even though these findings may be relevant to stress-related disorders, it is important to emphasise that the participants in this study were healthy, indicating that these are “normal” acute stress-induced changes in monocyte levels and receptor expression. Future studies should assess how acute stress affects monocyte levels in pathological chronic stress-related conditions, such as depression or posttraumatic stress disorders (Miller and Raison, 2016; Neigh and Ali, 2016; Reader et al., 2015; van de Wouw et al., 2019a).

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Author contributions

Van de Wouw M, Sichert M and Ritz N performed the flow cytometric analysis. Long-Smith C, Cusack AM and Berding K performed the SECPT. Van de Wouw M, Moloney G, Dinan TG and Cryan JF performed the data interpretation and wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbi.2021.03.005>.

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