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Authors	van de Wouw, Marcel;Lyte, Joshua M.;Boehme, Marcus;Sichetti, Marzia;Moloney, Gerard M.;Goodson, Michael S.;Kelley-Loughnane, Nancy;Dinan, Timothy G.;Clarke, Gerard;Cryan, John F.
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The Role of the Microbiota in Acute Stress-Induced Myeloid Immune Cell Trafficking

Marcel van de Wouw[1], Joshua M. Lyte[1#], Marcus Boehme[1], Marzia Sichetti[2], Gerard Moloney[1,3], Michael S. Goodson[4], Nancy Kelley-Loughnane[4], Timothy G. Dinan[1,5], Gerard Clarke[1,5], John F. Cryan[1,3]

[1]APC Microbiome Ireland, University College Cork, Cork, Ireland
[2]Unit of Biochemical Sciences and Health, Department of Pharmaceutical Sciences, University of Perugia, Italy
[3]Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland
[4]711[th] Human Performance Wing, Air Force Research Laboratory, Wright-Patterson Air Force Base, Dayton, Ohio, USA.
[5]Department of Psychiatry and Neurobehavioral Science, University College Cork, Cork, Ireland

[#]Present address: Poultry Production and Product Safety Research Unit, Agricultural Research Service, United States Department of Agriculture, Fayetteville, AR 72701, United States

Corresponding author:
J.F. Cryan: APC Microbiome Ireland; Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland. Email: j.cryan@ucc.ie.

Abstract

There has been a growing recognition of the involvement of the gastrointestinal microbiota in the development of stress-related disorders. Acute stress leads to activation of the neuroendocrine systems, which in turn orchestrate a large-scale redistribution of innate immune cells. Both these response systems are independently known to be primed by the microbiota, even though much is still unclear about the role of the gastrointestinal microbiota in acute stress-induced immune activation. In this study, we investigated whether the microbiota influences acute stress-induced changes in innate immunity using conventionally colonised mice, mice devoid of any microbiota (i.e. germ-free, GF), and colonised GF mice (CGF). We also explored the kinetics of stress-induced immune cell mobilisation in the blood, the spleen and mesenteric lymph nodes (MLNs). Mice were either euthanised prior to stress or underwent restraint stress and were then euthanised at various time points (i.e. 0, 45- and 240-minutes) post-stress. Plasma adrenaline and noradrenaline levels were analysed using ELISA and immune cell levels were quantified using flow cytometry. GF mice had increased baseline levels of adrenaline and noradrenaline, of which adrenaline was normalised in CGF mice. In tandem, GF mice had decreased circulating levels of LY6C^{hi} and LY6C^{mid}, CCR2+ monocytes, and granulocytes, but not LY6C-, CX3CR1+ monocytes. These deficits were normalised in CGF mice. Acute stress decreased blood LY6C^{hi} and LY6C^{mid}, CCR2+ monocytes while increasing granulocyte levels in all groups 45 minutes post-stress. However, only GF mice showed stress-induced changes in LY6C^{hi} monocytes and granulocytes 240 minutes post-stress, indicating impairments in the recovery from acute stress-induced changes in levels of specific innate immune cell types. LY6C-, CX3CR1+ monocytes remained unaffected by stress, indicating that acute stress impacts systemic innate immunity in a cell-type-specific manner. Overall, these data reveal novel cell-type-specific changes in the innate immune system in response to acute stress, which in turn are impacted by the microbiota. In conclusion,

the microbiota influences the priming and recovery of the innate immune system to an acute stressor and may inform future microbiota-targeted therapeutics aimed at modulating stress-induced immune activation in stress-related disorders.

Introduction

Chronic stress is a significant health concern for society, where it has been associated with various disease states including functional gastrointestinal disorders like irritable bowel syndrome (Stam et al. 1997, Moloney et al. 2016), and neuropsychiatric disorders like depression (Yang et al. 2015, Ramirez et al. 2017). Understanding the priming of the immune response (i.e. the exposure of a stimulus influencing the response to another later stimulus) to acute stress exposures is essential to understand and counteract the trajectory towards stress-related disorders.

Acute stress induces an elevation in systemic glucocorticoid (e.g. corticosterone) and catecholamine (e.g. adrenaline and noradrenaline) levels, which in turn orchestrate a large-scale redistribution of immune cells (Fauci et al. 1974, Steer et al. 1998, Morken et al. 2002, Dhabhar et al. 2012, Kim et al. 2014, Olnes et al. 2016, Yeager et al. 2016, van de Wouw et al. 2018). One of the key aspects of this immune cell redistribution are changes in myeloid cell numbers in the blood (Dhabhar et al. 2012), likely reflecting a migration of myeloid cells from their reservoir, or a migration into “immune privileged” tissues (e.g. lymph nodes, gut, brain) that are either at the interface between internal and external environments, or already inflamed (Dhabhar et al. 2012). Importantly, repeated exposure to acute stressors (i.e. chronic stress) results in systemic and central inflammation (Hansel et al. 2010, Rohleder 2014, Langgartner et al. 2018, Niraula et al. 2018). Monocytosis is a key aspect to this inflammation and has been implicated in gut-, and neuroinflammation, anxiety-like behaviour and anhedonia (Wohleb et al. 2013, Mackos et al. 2016, Zheng et al. 2016). As such, understanding how acute stress affects the innate immune system, in particular monocytes, will provide insight into the development of chronic stress and stress-related disorders.

It has long been known that the microbiota plays a pivotal role in the proper development and function of the innate immune system. For instance, proof-of-principle studies using mice devoid of any microbiota (i.e. germ-free, GF) have demonstrated that the microbiota is essential for the production of monocytes (Balmer et al. 2014, Khosravi et al. 2014, Karbach et al. 2016, Möhle et al. 2016). Furthermore, GF mice are more vulnerable to systemic infection with *Listeria monocytogenes* (Khosravi et al. 2014), and show a delayed clearance of pathogenic bacteria and a blunted immune response (Balmer et al. 2014). Research involving GF mice has also implicated a pivotal role for the microbiota in regulating stress-responses. Specifically, GF mice show an increased responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis to acute stress (Sudo et al. 2004, Clarke et al. 2013, Crumeyrolle-Arias et al. 2014, Luo et al. 2018), and have an increased expression of stress-related receptor pathway genes in the hippocampus (Luo et al. 2018). It is therefore not surprising that the microbiota-gut-brain axis has emerged as a key player in modulating monocyte trafficking (van de Wouw et al. 2019), and as a therapeutic target in ameliorating stress-related disorders (Lyte 2014, Lowry et al. 2016, Rea et al. 2016). As such, even though it is well-established that both the innate immune system and HPA-axis are primed by the microbiota, it is still unclear whether the gastrointestinal microbiota can influence acute stress-induced immune activation, which would have significant implications for reducing immune activation in stress-related disorders.

As such, we aimed to further investigate whether the microbiota influences catecholamine levels and stress-induced changes in monocyte subtype and granulocyte levels using GF mice and colonised GF mice. In tandem, we explored novel kinetics of stress-induced innate immune cell mobilisation in the peripheral circulation, spleens, and whether these immune cells traffic into mesenteric lymph nodes (MLNs).

Methodology

Ethical Approval

All experiments were conducted in accordance with European Directive 86/609/EEC. Animal Experimentation Ethics Committee and Health Products Regulatory Authority approvals were obtained before the start of animal-related experiments (#2016/014).

Animals

Breeding pairs (C57BL/6) were purchased from Taconic Biosciences (Germantown, NY, USA) and male F1-generation offspring (10-11 weeks of age) were used in this study. CON, GF and CGF mice were housed as 2-4 mice per cage and kept under a 12h light/dark cycle with identical environmental conditions of temperature (21 ± 1^{o}C) and humidity (55-60%). Animals were provided with the same autoclaved diet (Special Diets Services, Product code 801010, Essex, United Kingdom) and autoclaved water *ad libitum*. Conventional mice were reared and maintained in the standard animal facility in wire-top cages. GF mice were maintained in gnotobiotic flexible-film isolators. CGF mice were born and reared as GF mice until postnatal day 21, at which time mice were transferred from isolator housing to the standard animal facility and kept in wire-top cages lined with used-bedding from age- and sex-matched conventional mice.

Experimental Setup and Restraint Stress

Animals were randomly allocated to non-stress or stress groups, so each experimental group consisted of an n=7-9 per group (**Figure 1**). Samples sizes were calculated before the study was conducted. Within each stressed-group cage, mice were stressed in random order. Mice from non-stress cages were euthanised in a random sequence. All animal experiments were conducted between 0800h and 1400h. The same researcher conducted all animal handling to

lessen variability. Sterile polypropylene screw-cap 50 mL perforated conical tubes (Sarstedt, Nümbrecht, Germany) were used as the restraint device. New perforated conical tubes were used for each mouse. Separate rooms were used for the purposes of housing mice, subjecting mice to restraint stress, and sacrificing mice.

Restraint stress was performed by gently transferring a mouse from its home-cage to an identical but new cage with fresh bedding and moved into the room designated for restraint stress. The mouse was then carefully placed into the 50 mL perforated conical tube, the tube was laid horizontal and secured, and the mouse remained restrained for a single period of 15 minutes. Following 15 minutes of restraint stress, the mouse was immediately removed from restraint and then promptly transported to the cull room or transported back to their home-cage to recover from stress undisturbed for 45 minutes or 240 minutes, at which times mice were moved to the euthanization room. To control for transport stress, mice from the non-stress group were transported the same distance as stressed-group mice in identical but new cages with fresh bedding prior to entering the euthanization room.

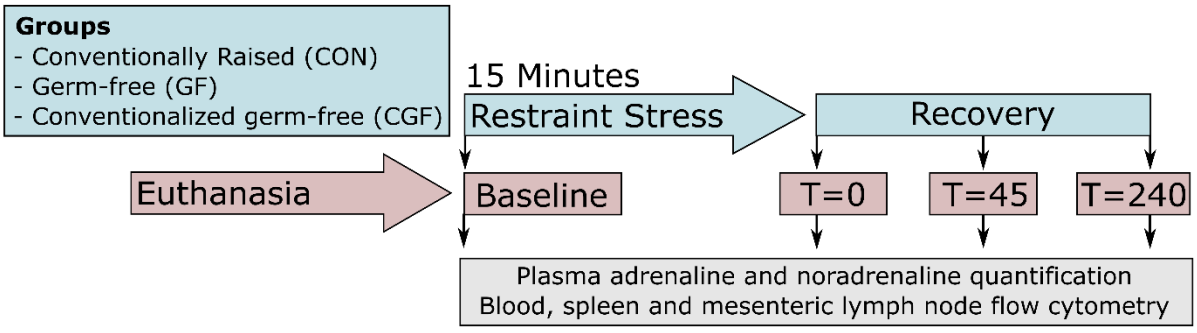

Figure 1. Experimental set-up. *Conventionally raised, germ-free and conventionalised germ-free mice were either sacrificed, or underwent restraint stress (15 minutes), after which they were allowed to recover (0, 45 and 240 minutes) and then sacrificed. Plasma was collected for adrenaline and noradrenaline quantification by ELISA. Blood, spleen and MLNs were collected to quantify myeloid cell populations by flow cytometry. Abbreviations: MLN = mesenteric lymph nodes.*

Tissue Collection

Upon entering the cull room, mice were immediately decapitated. Blood was collected and stored in a 3 mL EDTA-coated tube (Greiner bio-one, 454086). 50 µL was subsequently transferred into a new Eppendorf containing 2.5 µL 3% EDTA solution to prevent further blood clotting. This tube was centrifuged at 1500 g for 5 minutes at 4 °C, after which plasma was carefully transferred into a new Eppendorf tube and stored at -80 °C for later (nor)adrenaline quantification. The cell pellet was stored in wet ice and used for flow cytometry the same day. Spleens and mesenteric lymph nodes (MLNs) were dissected out of the animal, cleaned from fat tissue and stored in media (RPMI-1640 medium with L-glutamine and sodium bicarbonate (R8758, Sigma), supplemented with 10% FBS (F7524l, Sigma) and 1% Pen/strep (P4333, Sigma)) on wet ice for flow cytometry the same day.

Plasma Adrenaline and Noradrenaline Quantification

Plasma adrenaline and noradrenaline were quantified using an ELISA (Abnova, KA1877) according to the manufacturer's instructions with one minor modification, where 15 µL plasma was used instead of 300 µL. Absorbance was read at 450 nm using a Biotek Synergy H1 plate reader equipped with Gen5 software (Biotek, Winooski, VT, USA). The detection limit was 18 pg/mL for adrenaline and 93 pg/mL for noradrenaline.

Flow Cytometry

Flow cytometry was performed as previously described (Boehme et al. 2019, Gururajan et al. 2019). Briefly, blood cell pellets were resuspended in 10 mL home-made red blood cell lysis buffer (15.5 mM NH_4Cl, 1.2 mM $NaHCO_3$, 0.01 mM tetrasodium EDTA diluted in deionised water) for 3 minutes on ice. Samples were subsequently centrifuged at 1500 g for 5 minutes at 4 °C, aspirated and resuspended in 45 µL staining buffer (autoMACS Rinsing Solution

(Miltenyi, 130-091-222) supplemented with MACS BSA stock solution (Miltenyi, 130-091-376)) for the staining procedure. Splenocytes were isolated by flushing the spleen with media using a syringe. The cell suspension was subsequently centrifuged, aspirated and incubated with 1 mL lysis buffer (Sigma, R7757) for 5 minutes. 10 mL media was added to dilute the lysis buffer and the cell suspension was poured over a 70 µm strainer, after which it was centrifuged and aspirated. $2x10^6$ cells were resuspended in 90 µL staining buffer and split into 2 aliquots for the staining procedure. MLNs were transferred onto a 70 µm strainer and disassembled using the plunger of a 1 mL syringe. The strainer was subsequently rinsed with 10 mL media, and the cell suspension was centrifuged, aspirated, $2x10^6$ cells were resuspended in 90 µL staining buffer, and split into 2 aliquots for the staining procedure.

For the staining procedure, 5 µL of FcR blocking reagent (Miltenyi, 130-092-575) was added to each sample. Samples were subsequently incubated with a mix of antibodies (**Table 1**) for 30 minutes on ice, after which they were centrifuged, aspirated and fixed using 100 µL 4% PFA for 30 minutes on ice. Samples were finally centrifuged, aspirated and resuspended in staining buffer for flow cytometric analysis the following day on the BD FACSCalibur. Data were analysed using FlowJo (Version 10). Cell populations were selected as following: blood/splenic LY6C^{hi} Monocytes: CD11b+, LY6C^{hi}; blood/spleen LY6C^{mid} Monocytes: CD11b+, LY6C^{mid}, SSC^{low}, CCR2+; blood/splenic LY6C^{low} Monocytes: CD11b+, LY6C-, SSC^{low}, CX3CR1+; blood/splenic Granulocytes: CD11b+, SSC^{hi}; splenic Macrophages: CD11b+, LY6G-, MHC-II+, F4/80+; MLN LY6C^{hi} Monocytes: CD11b+, LY6C+, LY6G-; MLN Neutrophils: CD11b+, LY6C+, LY6G+; MLN Macrophages: CD11b+, LY6C-, F4/80+; MLN Dendritic cells: CD11c+, MHC-II+. Gating strategies for monocytes and granulocytes are depicted in **Figure 3**, **Figure 5** and **sFigure 1**. Gating strategies for macrophages and dendritic cells are depicted in **sFigure 3** and **sFigure 5**. Expression of the LY6C, CCR2 and

CX3CR1 receptors on monocyte subtype were consistent with previous reports (Ginhoux et al. 2014, Yang et al. 2014) (**sFigure 2**). The investigated cell populations were normalised to overall cell levels. Experimenters performing flow cytometry were blind to group assignments.

Table 1: Mixes of antibodies used for all tissues and target populations.

Receptor	Conjugation	Volume per sample (µL)	Company and product number
Monocyte and granulocyte panel for blood and spleen			
CD11b	VioBright™ FITC	2	Miltenyi 130-109-290
LY6C	PE	2	Miltenyi 130-102-391
CX3CR1	PerCP-Cy5.5	0.3	Biolegend 149010
CCR2	APC	5	Miltenyi 130-108-723
Macrophage panel for spleen			
CD11b	VioBright™ FITC	2	Miltenyi 130-109-290
MHC-II	PE	5	Miltenyi 130-102-186
LY6G	PerCP-Vio700	5	Miltenyi 130-107-917
F4/80	APC	5	Miltenyi 130-102-379
Monocyte, neutrophil and macrophage panel for MLNs			
CD11b	VioBright™ FITC	2	Miltenyi 130-109-290
LY6C	PE	2	Miltenyi 130-111-778
LY6G	PerCP-Vio700	5	Miltenyi 130-107-917
F4/80	APC	5	Miltenyi 130-102-379
Dendritic cell panel for MLNs			
CD11c	PE	2	Miltenyi 130-110-838
MHC-II	APC	5	Miltenyi 130-102-139

Statistical Analysis

Data were assessed for normality using Shapiro-Wilk test and Levene's test for equality of variances and were deemed non-parametric if any of the two tests were significant (<0.05), as n-numbers were unequal between groups. All data of this study were non-parametric. Baseline differences between conventionally-raised mice (CON), GF and conventionalised germ-free (CGF) groups were analysed using the Kruskal-Wallis test, followed by the Mann-Whitney U test. To first investigate whether stress impacted immune cell levels overall, we used a Kruskal-Wallis test, which was followed by Mann-Whitney U tests on individual time points post-stress for each group/microbiota condition if a significant stress effect was present. All post-stress data from animals were normalised to baseline data (pre-stress) to ensure that figures were interpretable, as baseline levels of immune cell levels differed manifold between groups. Statistical analysis was performed using SPSS software version 26 (IBM Corp). Values that were two standard deviations from the mean were considered outliers and excluded from the analysis. Data are expressed as boxplots. A p-value <0.05 was deemed significant.

Results

Adrenaline and noradrenaline levels are affected by microbiota status

As the catecholamines adrenaline and noradrenaline have previously been reported to induce immune cell trafficking (Dhabhar et al. 2012), we first quantified plasma adrenaline and noradrenaline levels. There was a significant effect of microbiota status on both adrenaline and noradrenaline levels ($X^2(2) = 6.385$, $p = 0.041$; $X^2(2) = 9.242$, $p = 0.010$). Specifically, GF mice have elevated plasma levels of adrenaline and noradrenaline ($p = 0.039$, $p = 0.005$), of which only adrenaline was normalised in response to colonisation of the microbiota in CGF mice ($p = 0.039$) (**Figure 2**). No significant stress-induced changes in adrenaline and noradrenaline levels were observed.

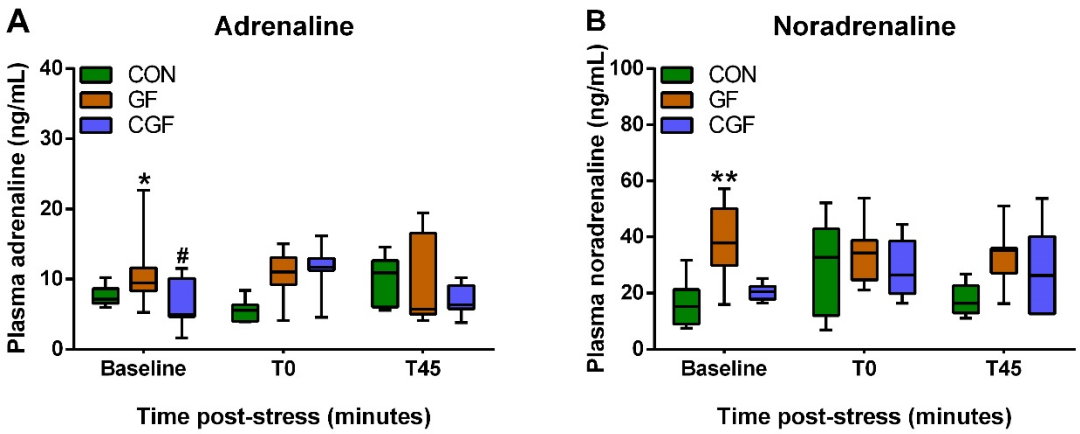

Figure 2. Absence of a microbiota results in elevated circulating adrenaline and noradrenaline levels, while colonisation of the microbiota ameliorates adrenaline levels. *Plasma adrenaline and noradrenaline levels were quantified before (baseline) and after stress (T0 and T45) (A, B). No statistically significant effect was observed by stress. Data were non-normally distributed and analysed using the Mann-Whitney U test. Significant differences are depicted as: *p < 0.05 and **p < 0.01 compared to CON; #p < 0.05 compared to GF. Data are presented as boxplots (n = 7-9). Abbreviations: CON = Conventionally raised; GF = Germ-free; CGF = Conventionalized germ-free.*

Absence of microbiota results in deficits in myeloid cells levels, which are ameliorated upon colonisation of the microbiota

Analysis of blood myeloid cell levels revealed an effect of microbiota status on $LY6C^{hi}$ and $LY6C^{mid}$, CCR2+ monocytes ($X^2(2) = 16.541$, $p < 0.001$; $X^2(2) = 11.351$, $p = 0.003$), but not levels of LY6C-, CX3CR1+ monocytes (**Figure 3B-D**). These changes were explained by GF mice having deficits in $LY6C^{hi}$ and $LY6C^{mid}$, CCR2+ monocytes ($p < 0.001$; $p = 0.014$), which were normalized in CGF mice ($p < 0.001$). There was also an effect of the microbiota on circulating granulocyte levels ($X^2(2) = 12.279$, $p = 0.002$) (**Figure 3E**). Here, granulocyte levels were decreased in GF mice ($p = 0.026$), which were restored in CGF mice ($p < 0.001$). Similar to the blood immune cell profile, microbiota status affected splenic $LY6C^{hi}$ and $LY6C^{mid}$, CCR2+ monocyte levels ($X^2(2) = 11.021$, $p = 0.004$; $X^2(2) = 9.890$, $p = 0.007$) (**Figure 3F, G**). Specifically, GF mice had decreased cell levels ($p = 0.005$; $p = 0.006$), which subsequently increased in CGF mice ($p = 0.059$; $p = 0.006$). No differences in splenic LY6C-, CX3CR1+ monocyte and granulocyte levels were observed (**Figure 3H, I**). Splenic macrophage levels were decreased in both GF and CGF mice (**sFigure 3B, C**).

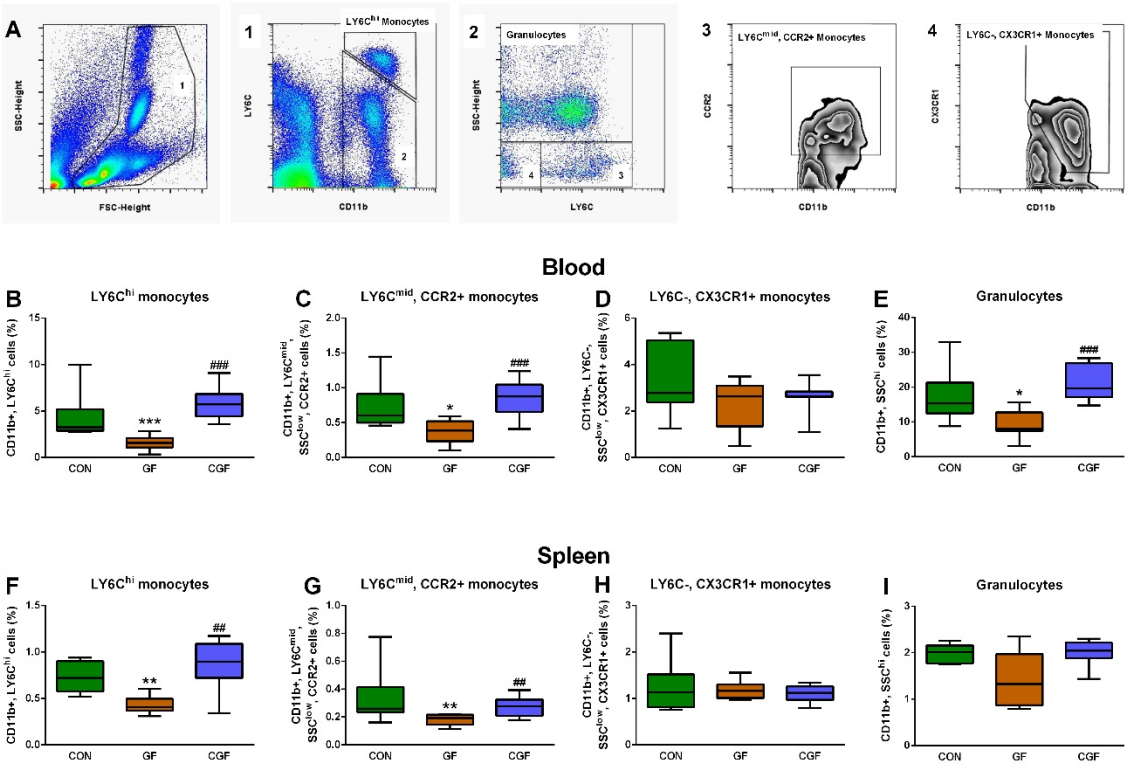

Figure 3. Absence of microbiota results in deficits in circulating blood and splenic myeloid cell levels, which are ameliorated by colonisation with microbiota. *Gating was performed as following (A). Cells were selected based on FSC-height and SSC-height. 1) LY6C^{hi} monocytes (CD11b+, LY6C^{hi}) were subsequently selected, whereas CD11b+, LY6C^{low}/- cells were used to select other populations. 2) Granulocytes (CD11b+, SSC^{hi}) were then selected. 3) While SSC^{hi}, LY6C- cells were used to gate LY6C^{mid}, CCR2+ monocytes (CD11b+, SSC^{low}, LY6C^{mid}, CCR2+). 4) SSC^{hi}, LY6C- cells were used to gate LY6C-, CX3CR1+ monocytes (CD11b+, SSC^{low}, LY6C-, CX3CR1+). LY6C^{hi} monocyte, LY6C^{mid}, CCR2+ monocyte, LY6C-, CX3CR1+ monocyte and granulocyte levels were quantified in both blood (B-E) and the spleen (F-I). Data were non-normally distributed and analysed using the Mann-Whitney U test. Significant differences are depicted as: *p < 0.05, **p < 0.01 and ***p < 0.001 compared to CON; $^{\#}$p < 0.05, $^{\#\#}$p < 0.01 and $^{\#\#\#}$p < 0.001 compared to GF. Data are presented as boxplots (n = 7-9). Abbreviations: CON = Conventionally raised; GF = Germ-free; CGF = Conventionalized germ-free.*

Stress induces changes in peripheral myeloid cell levels, which are impacted by microbiota status

Stress-induced significant changes in circulating LY6C^{hi} monocytes ($\chi^2(1) = 10.610$, $p = 0.001$) (**Figure 4A**). Specifically, stress-reduced blood LY6C^{hi} monocytes levels 45 minutes post-stress (CON: $p < 0.001$; GF: $p = 0.054$; CGF: $p < 0.001$), which was normalized in CON mice 240 minutes post-stress, but not GF and CGF mice ($p = 0.054$; $p = 0.006$). Similarly, stress also affected LY6C^{mid}, CCR2+ monocytes ($\chi^2(1) = 5.038$, $p = 0.025$) (**Figure 4B**). Here, there were decreased levels of LY6C^{mid}, CCR2+ monocytes 45 minutes post-stress in CON and CGF mice ($p = 0.004$; $p = 0.023$), but not GF mice. These stress-induced changes were still present in CGF mice 240 minutes post-stress ($p < 0.046$). Stress also significantly impacted circulating granulocyte levels ($\chi^2(1) = 6.403$, $p = 0.011$) (**Figure 4C**). Stress decreased cell levels in CON mice immediately after stress ($p = 0.021$), whereas granulocytes were increased in GF mice ($p = 0.038$). 45-minutes post-stress, circulating granulocyte levels were increased in all groups (CON: $p = 0.004$; GF: $p < 0.001$; CGF: $p < 0.001$), which were still elevated in GF mice only ($p = 0.017$). In the spleen, we observed similar changes in LY6C^{hi} monocytes compared to the peripheral circulation, where stress impacted cell levels ($\chi^2(1) = 8.528$, $p = 0.003$) (**Figure 4D**). Specifically, LY6C^{hi} monocytes were decreased immediately after stress in CON and GF mice ($p = 0.043$; $p = 0.006$), which were additionally decreased 240 minutes post-stress in GF mice ($p < 0.001$). No stress-induced effects on splenic LY6C^{mid}, CCR2+ monocyte and granulocyte cell levels) (**Figure 4E, F**). Finally, no circulating and splenic stress-induced differences were observed in LY6C-, CX3CR1+ monocytes in any of the groups (**sFigure 4A**).

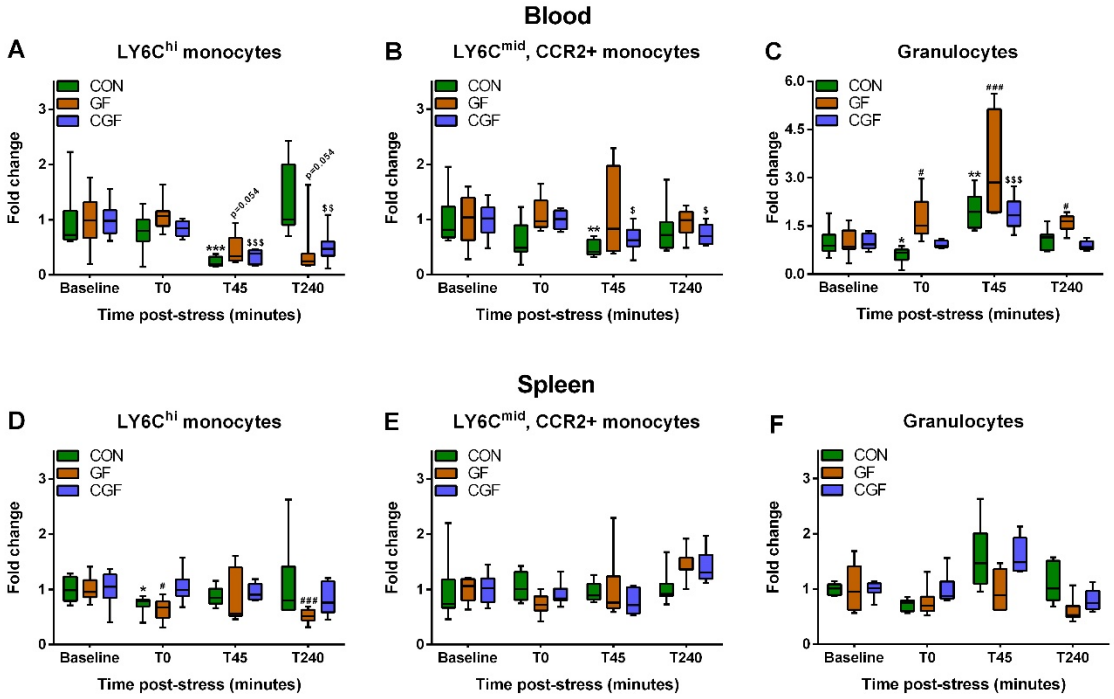

Figure 4. Stress induced circulating and splenic myeloid cell trafficking, which is affected by microbiota status. *LY6C^{hi} monocyte, LY6C^{mid}, CCR2+ monocyte and granulocte levels were quantified before and after acute stress in the blood (A-C), and spleen (D-F). Data were non-normally distributed and analysed using the Mann-Whitney U test. Significant differences are depicted as: *p < 0.05, **p < 0.01 and ***p < 0.001 compared to baseline for CON mice; $^{\#}$p < 0.05 and $^{\#\#\#}$p < 0.001 compared to baseline for GF mice. $^{\$}$p < 0.05, $^{\$\$}$p < 0.01 and $^{\$\$\$}$p < 0.001 compared to baseline for CGF mice. Post-stress data from animals were normalized to baseline data (pre-stress). Data are presented as boxplots (n = 7-9) Abbreviations: CON = Conventionally raised; GF = Germ-free; CGF = Conventionalized germ-free.*

Even though myeloid cell levels in MLNs remain affected by microbiota status, stress does not induce myeloid cell trafficking into MLNs

We subsequently hypothesised that one of the target tissues to which myeloid cells traffic, could be MLNs (Dhabhar et al. 2012), considering their crucial role in pathogen defence (Gasteiger et al. 2016). Similar to the blood and spleen, we observed an effect of the microbiota on LY6C+ monocytes and neutrophils, the major cell population of granulocytes (LY6C+ monocytes: $X^2(2) = 9.041$, neutrophils: $p = 0.011$; $X^2(2) = 5.536$, $p = 0.063$). Specifically, GF mice had deficits in LY6C+ monocytes and neutrophils ($p = 0.029$; $p = 0.040$), which were ameliorated in CGF mice ($p = 0.006$; $p = 0.059$) (**Figure 5B, D**). However, stress did not significantly impact monocyte and neutrophil levels in MLNs (**Figure 5C, E**). We subsequently hypothesised that the absence of differences in monocytes in MLNs post-stress could be explained by these cells differentiating into macrophages or dendritic cells but observed no differences in these cell populations in response to stress (**sFigure 5C, E**).

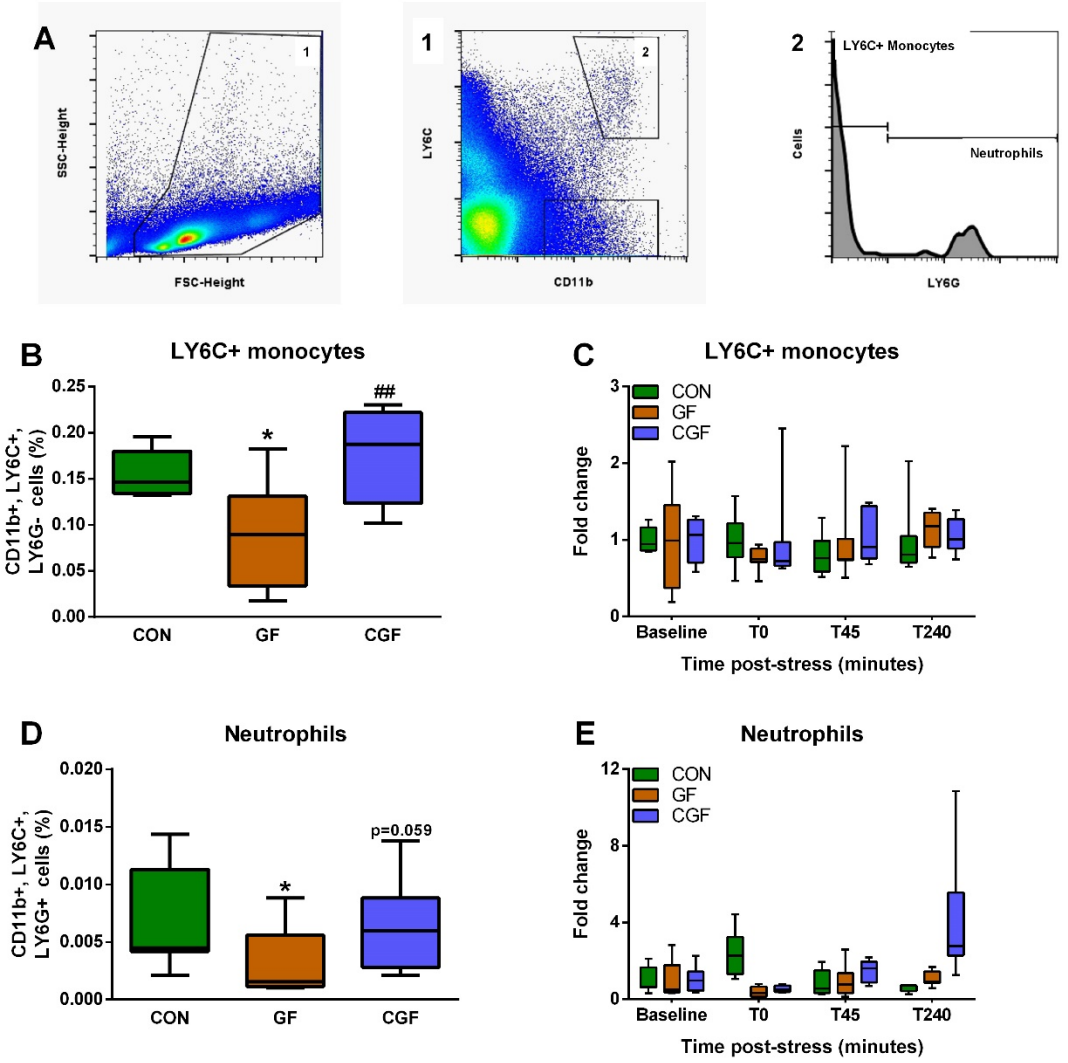

Figure 5. Absence of a microbiota reduced MLN monocyte and neutrophil levels, which are ameliorated by colonisation of the microbiota but unaffected by stress. *Gating was performed as following (A). Cells were selected based on FSC-height and SSC-height. 1) CD11b+, LY6C cells were selected. 2) this was followed by the selection of LY6C+ monocytes (CD11b+, LY6C+, LY6G-) and neutrophils (CD11b+, LY6C+, LY6G+). LY6C+ monocyte levels were quantified at baseline (B) and in response to stress (C). Neutrophil levels were also quantified at baseline (D) and in response to stress (E). Data were non-normally distributed and analysed using the Mann-Whitney U test. Significant differences are depicted as: *$p < 0.05$ compared to CON; $^{\#\#}p < 0.01$ compared to GF mice. Post-stress data from animals were normalised to baseline data (pre-stress). Data are presented as boxplots (n = 7-9). Abbreviations: CON = Conventionally raised; GF = Germ-free; CGF = Conventionalized germ-free; MLN = mesenteric lymph node.*

Discussion

There has been increased attention on the role of the immune system in stress-responsiveness and the development of stress-related disorders (Ramirez et al. 2017, Bobel et al. 2018, Langgartner et al. 2018). In the present study, we found, for what is to our knowledge the first time, that the microbiota modulates circulating adrenaline and noradrenaline levels, as well as myeloid cell levels in a cell subtype-specific manner, indicating that the microbiota is able to contribute to the priming of the immune system to acute stressors. Furthermore, we report that acute stress modulates the innate immune system in a cell type-specific manner that is regulated by the microbiota.

The absence of microbiota resulted in elevated levels of circulating adrenaline and noradrenaline, of which only adrenaline was normalised upon colonisation of the microbiota, which has not been reported before. No statistically significant differences were observed in plasma adrenaline and noradrenaline levels in response to acute stress, meaning that these catecholamine data should be interpreted with care. Nonetheless, it has been reported that GF mice have decreased levels of noradrenaline in the gut lumen (Asano et al. 2012, Sudo 2019), which has been implicated in host-microbe crosstalk in conditions of stress (Lyte et al. 2011). Furthermore, GF mice have exaggerated elevations in circulating corticosterone and adrenocorticotropic hormone in response to an acute stressor, even though basal levels remain unaffected (Sudo et al. 2004, Clarke et al. 2013, Crumeyrolle-Arias et al. 2014, Luo et al. 2018). Alterations in basal and stress-induced increases in catecholamines and glucocorticoids have been linked to the priming of the immune system to stress (Sapolsky et al. 2000, Sorrells et al. 2010). Along with the changes in baseline catecholamine levels, there were deficits in circulating, splenic and MLN myeloid cell populations in GF mice. Subsequent colonisation of the microbiota normalised these deficits. This novel observation is consistent with previous

findings showing a decreased myelopoiesis in GF and antibiotic-treated mice (Balmer et al. 2014, Khosravi et al. 2014, Möhle et al. 2016). We additionally show that this deficit in monocyte levels is subtype-specific, as no differences in LY6C-, CX3CR1+ monocytes were observed in GF mice. This is quite surprising, as LY6C^{hi} monocytes, which were decreased in GF mice, give rise to LY6C-, CX3CR1+ monocytes (Ginhoux et al. 2014). This might indicate that the microbiota influences the production of LY6C^{hi} monocytes, while the subsequent differentiation into LY6C-, CX3CR1+ is regulated by other processes. It is also interesting to point out that LY6C-, CX3CR1+ are involved in the surveillance of the vessel wall within the vasculature for injury, whereas LY6C^{hi} monocytes are recruited at sites of inflammation where they differentiate into dendritic cells and macrophages (Ginhoux et al. 2014). These deficits in LY6C^{hi} monocytes are in line with reports that GF mice are more vulnerable to systemic infection with *Listeria monocytogenes* (Khosravi et al. 2014). Similarly, GF mice show a delayed clearance of pathogenic bacteria, which is paired with a blunted immune response (Balmer et al. 2014).

In conventional mice, our results also reveal novel innate immune cell-type-specific changes induced by acute stress, where there is a decrease in LY6C^{hi} and LY6C^{mid}, CCR2+ monocytes, while LY6C-, CX3CR1+ monocytes remained unaffected. These specific innate immune cell-type-specific changes might be highly relevant to the development of chronic stress-associated impairments. For instance, the LY6C^{hi} monocyte subtype is often associated with inflammation (Ginhoux et al. 2014), which in turn has been linked to stress-related disorders (Hansel et al. 2010, Rohleder 2014, Langgartner et al. 2018, Niraula et al. 2018). Furthermore, in line with our findings showing that acute stress changes monocyte levels in a subtype specific manner, chronic psychosocial stress results in elevated levels of blood LY6C^{hi} monocytes, whereas LY6C- monocyte levels remain unaffected (Gururajan et al. 2019). Chronic stress is also

associated with increased levels of splenic monocytes (Engler et al. 2004, McKim et al. 2018), which can be deployed to sites of inflammation (Swirski et al. 2009). Our data also reveals, for the first time to our knowledge, that acute stress decreases LY6C^{hi} monocytes in the splenic reservoir, potentially indicating a mobilization of monocytes from their reservoir. Notable, our splenic data is not paralleled by an increase in blood monocyte levels, which might be explained by more monocytes migrating into target tissues than the number of splenic monocytes being released. More research is warranted to validate this hypothesis. Overall, it seems that these monocyte subtype-specific changes could be highly relevant to the development of chronic stress-associated impairments. It is therefore important to note that chronic stress-associated monocytosis has been implicated in gut-, and neuroinflammation, anxiety-like behaviour and anhedonia (Wohleb et al. 2013, Mackos et al. 2016, Zheng et al. 2016).

We also observe an increase in circulating granulocyte levels in response to stress. Increased cell-specific numbers in the peripheral circulation likely reflect a mobilisation of immune cells from certain compartments, while decreases in cell-specific numbers reflect that these cells traffic into target organs or sites of immune activation (Dhabhar et al. 2012). For instance, acute stress has shown to induce leukocyte trafficking into the skin (Dhabhar et al. 1996). Increased neutrophil levels in response to restraint stress have previously been reported (Dhabhar et al. 2012). Notably, Dhabhar and colleagues also reported decreased blood monocyte levels in response to acute stress, which was statistiscally insignificant, even though they reported in their discussion that their study was underpowered (Dhabhar et al. 2012). So these results should be interpreted with caution. Finally, cold water-induced stress decreases circulating monocyte levels in healthy male volunteers (Brazaitis et al. 2014).

We hypothesized that MLNs could be one of those target organs, as acute and chronic stress increase the permeability of the intestinal barrier allowing intestinal bacteria to translocate to

MLNs (Ferrier et al. 2003, Scott et al. 2017, van de Wouw et al. 2018), where monocyte-derived macrophages play a crucial role in pathogen defence (Gasteiger et al. 2016). However, no differences in LY6C^{hi} monocyte and neutrophil levels were observed in response to stress. Similarly, no differences were observed in MLN dendritic cell and macrophage levels, indicating that the absence of stress-induced differences cannot be explained by monocytes differentiating into these cell types. As such, more research is warranted to understand which tissues monocytes traffic in response to an acute stressor (e.g., brain, skin). It is important to note that acute stress induces leukocyte trafficking into the skin (Dhabhar et al. 1996), whereas chronic stress is associated with increased levels of LY6C^{hi} monocytes in the brain (Wohleb et al. 2013, Zheng et al. 2016).

Our data also reveals that the innate immune system of mice with a different microbiota status respond differentially to acute stress. Both CON and CGF mice have decreased blood LY6C^{mid}, CCR2 monocyte levels 45 minutes after stress, which is not observed in GF mice. This might indicate a failure of this particular aspect of the immune system to respond to an acute stressor. Such differences could be related to the increase in basal noradrenaline levels observed in GF mice, which might prime these monocytes to respond differentially to stimuli (Sapolsky et al. 2000). It is also interesting to note that circulating granulocytes were increased in GF mice immediately after stress, which was not observed in CON and CGF mice. This could indicate an oversensitivity of granulocytes to respond to acute stress in the absence of a microbiota. The status of the microbiota also influenced the recovery of the innate immune system after an acute stressor. Acute stress decreased blood LY6C^{hi} monocytes 45 minutes post-stress in all groups, but these changes were still present in GF and CGF mice after 240 minutes. Similarly, splenic LY6C^{hi} monocytes were immediately decreased after acute stress in CON and GF mice and

were also decreased 240 minutes post-stress in GF mice. Finally, the acute stress-induced increases in blood granulocytes persisted 240 minutes post-stress in GF mice only.

Taken together, our data reveal novel cell-type-specific interactions between acute stress and the innate immune system that are regulated by the microbiota. It will be of value for future studies to investigate the impact of stress on the gut microbiome over a longer period of time (Bailey et al. 2011, Bharwani et al. 2016, Burokas et al. 2017, Dunphy-Doherty et al. 2018). Future studies focused on develop microbiota-targeted therapeutics to modulating stress-induced innate immune activation are now warranted.

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Author Contributions

J.M.L. performed the restraint stress. J.M.L. and M.vd.W. performed the ELISAs. M.vd.W., M.B., M.S., and G.M. performed flow cytometry. M.vd.W. did the analysis and interpretation of the flow cytometry data. M.vd.W., J.M.L., G.C., T.G.D. and J.F.C. conceived and contributed to the experimental design. M.vd.W. drafted the initial manuscript. All authors edited, reviewed, and approved the manuscript.

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Figure legends

Figure 1. Experimental set-up. Conventionally raised, germ-free and conventionalised germ-free mice were either euthanised, or underwent restraint stress (15 minutes), after which they were allowed to recover (0, 45 and 240 minutes) and then euthanised. Plasma was collected for adrenaline and noradrenaline quantification by ELISA. Blood, spleen and MLNs were collected to quantify myeloid cell populations by flow cytometry. Abbreviations: MLN = mesenteric lymph nodes.

Figure 2. Absence of a microbiota results in elevated circulating adrenaline and noradrenaline levels, while colonisation of the microbiota ameliorates adrenaline levels. Plasma adrenaline and noradrenaline levels were quantified before (baseline) and after stress (T0 and T45) (A, B). No statistically significant effect was observed by stress. Data were non-normally distributed and analysed using the Mann-Whitney U test. Significant differences are depicted as: *$p < 0.05$ and **$p < 0.01$ compared to CON; $^{\#}p < 0.05$ compared to GF. Data are presented as boxplots (n = 7-9). Abbreviations: CON = Conventionally raised; GF = Germ-free; CGF = Conventionalized germ-free.

Figure 3. Absence of microbiota results in deficits in circulating blood and splenic myeloid cell levels, which are ameliorated by colonisation with microbiota. Gating was performed as following (A). Cells were selected based on FSC-height and SSC-height. 1) $LY6C^{hi}$

monocytes (CD11b+, LY6C^{hi}) were subsequently selected, whereas CD11b+, LY6C^{low}/- cells were used to select other populations. 2) Granulocytes (CD11b+, SSC^{hi}) were then selected. 3) While SSC^{hi}, LY6C- cells were used to gate LY6C^{mid}, CCR2+ monocytes (CD11b+, SSC^{low}, LY6C^{mid}, CCR2+). 4) SSC^{hi}, LY6C- cells were used to gate LY6C-, CX3CR1+ monocytes (CD11b+, SSC^{low}, LY6C-, CX3CR1+). LY6C^{hi} monocyte, LY6C^{mid}, CCR2+ monocyte, LY6C-, CX3CR1+ monocyte and granulocyte levels were quantified in both blood (B-E) and the spleen (F-I). Data were non-normally distributed and analysed using the Mann-Whitney U test. Significant differences are depicted as: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to CON; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to GF. Data are presented as boxplots (n = 7-9). Abbreviations: CON = Conventionally raised; GF = Germ-free; CGF = Conventionalized germ-free.

Figure 4. Stress induced circulating and splenic myeloid cell trafficking, which is affected by microbiota status. LY6C^{hi} monocyte, LY6C^{mid}, CCR2+ monocyte and granulocte levels were quantified before and after acute stress in the blood (A-C), and spleen (D-F). Data were non-normally distributed and analysed using the Kruskall Wallis test followed by the Mann-Whitney U test. Significant differences are depicted as: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to baseline for CON mice; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ compared to baseline for GF mice. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ and $^{\$\$\$}p < 0.001$ compared to baseline for CGF mice. Post-stress data from animals were normalized to baseline data (pre-stress). Data are presented as boxplots (n = 7-9) Abbreviations: CON = Conventionally raised; GF = Germ-free; CGF = Conventionalized germ-free.

Figure 5. Absence of a microbiota reduced MLN monocyte and neutrophil levels, which are ameliorated by colonisation of the microbiota but unaffected by stress. Gating was performed as following (A). Cells were selected based on FSC-height and SSC-height. 1)

CD11b+, LY6C cells were selected. 2) this was followed by the selection of LY6C+ monocytes (CD11b+, LY6C+, LY6G-) and neutrophils (CD11b+, LY6C+, LY6G+). LY6C+ monocyte levels were quantified at baseline (B) and in response to stress (C). Neutrophil levels were also quantified at baseline (D) and in response to stress (E). Data were non-normally distributed and analysed using the Mann-Whitney U test. Significant differences are depicted as: *$p < 0.05$ compared to CON; $^{\#\#}p < 0.01$ compared to GF mice. Post-stress data from animals were normalised to baseline data (pre-stress). Data are presented as boxplots (n = 7-9). Abbreviations: CON = Conventionally raised; GF = Germ-free; CGF = Conventionalized germ-free; MLN = mesenteric lymph node.

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