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Glucocorticoids Enhance the *In Vivo* Migratory Response of Human Monocytes

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Abstract

Glucocorticoids (GCs) are best known for their potent anti-inflammatory effects. However, an emerging model for glucocorticoid (GC) regulation of *in vivo* inflammation also includes a delayed, preparatory effect that manifests as enhanced inflammation following exposure to an inflammatory stimulus. When GCs are transiently elevated *in vivo* following exposure to a stressful event, this model proposes that a subsequent period of increased inflammatory responsiveness is adaptive because it enhances resistance to a subsequent stressor. In the present study, we examined the migratory response of human monocytes/macrophages following transient *in vivo* exposure to stress-associated concentrations of cortisol. Participants were administered cortisol for 6 hours to elevate *in vivo* cortisol levels to approximate those observed during major systemic stress. Monocytes in peripheral blood and macrophages in sterile inflammatory tissue (skin blisters) were studied before and after exposure to cortisol or placebo. We found that exposure to cortisol induced transient upregulation of monocyte mRNA for CCR2, the receptor for monocyte chemotactic protein-1 (MCP-1/CCL2) as well as for the chemokine receptor CX3CR1. At the same time, mRNA for the transcription factor I κ B α was decreased. Monocyte surface expression of CCR2 but not CX3CR1 increased in the first 24 hours after cortisol exposure. Transient exposure to cortisol also led to an increased number of macrophages and neutrophils in fluid derived from a sterile inflammatory site *in vivo*. These findings suggest that the delayed, pro-inflammatory effects of cortisol on the human inflammatory responses may include enhanced localization of effector cells at sites of *in vivo* inflammation.

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Keywords

cortisol; stress; monocyte; macrophage; CCR2; CX3CR1; IκBα

1. INTRODUCTION

Glucocorticoids (GCs) are widely understood to suppress inflammation through regulation of signaling pathways in leukocytes and other effector cells. Until recently, these potent and clinically important effects of GCs have obscured results from earlier research that demonstrated GC support and even stimulation of *in vivo* defense mechanisms¹. An emerging model for GC regulation of defense mechanisms includes not only their well-known anti-inflammatory properties but also a teleological conception in which GCs can enhance the *in vivo* response to external stressors. Evidence for GC enhancement of inflammation was described as early as 1954 by Selye², was reintroduced into the clinical literature in 1984³, and has since been expanded by subsequent research^{4–10}. Time is a critical variable. Concurrent exposure of immune effector cells to GCs engenders the two best-known properties of GCs: 1) At low ‘basal’ concentrations, a ‘permissive’ GC effect supports other metabolic and inflammatory processes so that they proceed normally^{11,12}. The absence of this GC activity leads to the Addisonian crisis first described in the 19th century¹³. 2) At higher concentrations of GCs, when concurrent with systemic stress, the well-known anti-inflammatory effects of GCs are observed and act to prevent damage from an excessive inflammatory response to an imposed stimulus¹⁴. However, markedly different effects can be observed when *transient* (hours) *in vivo* exposure to elevated GC concentrations *is followed by a return to normal GC concentrations*: 1) ‘Preparatory’ effects are observed in which cells, especially immune effector cells, undergo transcriptional and phenotypic changes that prime them for an enhanced response to a subsequent immune stimulus^{4,6,15–18}. These preparatory effects have been reported for both innate (especially following TLR4 ligation^{6–8,18}) and adaptive immune cells^{8,15,19–22}. 2) Stimulation of effector cell responses is observed if an external inflammatory stimulus is applied during the preparatory period^{4,9,17,23,24}. Preparatory and stimulatory effects typically manifest beginning several hours after a transient exposure to elevated GCs (and a subsequent return to normal GC concentrations) and may last for up to a week^{4,18}. These delayed preparatory and stimulatory effects appear to enhance an organism’s resistance to a subsequent stressful event (infection after injury, for example). The teleological components of this model therefore include the initial response of an organism to an external stimulus resulting in elevated GCs that enable defense mechanisms to proceed optimally by a permissive effect and then act to prevent an exaggerated inflammatory response from damaging normal tissue by way of GC anti-inflammatory effects. The following period of enhanced inflammatory responsiveness appears to be an adaptive effect that promotes resistance to subsequent stressors.

In vivo experiments in humans and animals support this concept. Barber et al showed that a 6-hour *in vivo* exposure of healthy humans to pharmacological doses of cortisol induces a substantial increase in their pro-inflammatory response to a subsequent challenge with bacterial endotoxin⁴. This same effect can be reproduced *in vivo* by “stress cortisol”

concentrations that are similar to those observed during a systemic stress such as surgery²⁴. Animal experiments yield similar results. Exposure of rats to the sterile stress of a skin burn induces a subsequent state of resistance to an otherwise lethal inoculation with gram-negative bacteria and enhances the pro-inflammatory response to a sublethal injection of bacterial lipopolysaccharide^{25,26}. Systemic stress can also induce expression of Toll-like receptors 4 (TLR4) and 2 (TLR2) on macrophages^{19,26} suggesting that stress may increase resistance to bacterial infection through up-regulation of the proinflammatory response to bacterial pathogen-associated molecular patterns (PAMPs). Enhanced activation of anti-viral responses has also been reported in mice subjected to repeated social stress²⁷. In these studies, the relative contribution of GCs and other stress responding hormones, specifically catecholamines, has still to be clarified²⁸. GCs have also been shown to enhance neuroinflammatory responses in animals in association with an upregulation of high-mobility group box-1 (HMGB1), which in turn primes the inflammasome response through TLR2/TLR4 signaling²⁹. *In vitro*, murine bone marrow-derived macrophages^{18,30} and peritoneal macrophages³¹ show a similar response pattern of a GC-induced increase in the pro-inflammatory response to PAMPs.

Until recently, there have been few reports on the cellular and molecular events that are associated with GC stimulation of human immune responses. In the experiments described here we demonstrate that GCs can enhance key components of the human monocyte/macrophage migratory response.

2. MATERIALS AND METHODS

2.1 Clinical protocols

Clinical protocols were approved by the Dartmouth College Committee for the Protection of Human Subjects (Institutional Review Board) and written informed consent was obtained from all participants. Subjects were healthy non-obese males or females, nonsmokers, taking no chronic medication and with no history of illness or injury within the 3 months prior to study.

2.1.1 *In vivo* stress cortisol exposure—Participants were exposed to stress-associated concentrations of cortisol for 6 hours initially by the intravenous and subsequently the oral route. Intravenous cortisol (hydrocortisone; SoluCortef, Upjohn) was administered at a rate of 1.5ug/kg/min, a dose that we have previously shown increases plasma cortisol to concentrations that are observed when humans are exposed to a systemic stress such as major surgery³² or endotoxemia²⁴. Oral cortisol (Cortef®, Pfizer) was administered as a 30mg oral loading dose followed by 10 mg orally every hour for 5 subsequent doses leading to similar increases of *in vivo* cortisol concentrations as described below.

2.1.2 Sterile inflammation—Healthy volunteers (n=7) participated in the following protocol: From 0800 to 0930, eight 8mm diameter blisters were raised on the volar aspect of a participant's forearm using a commercially available device (NP-4 Negative Pressure Instrument, Electronic Diversities, Finksburg, MD). The device has a metal plate with 8mm diameter holes that is placed against the skin and attached to a negative pressure chamber with programmable suction. A negative pressure of -250 mmHg is applied to the chamber

over the skin. After 90 minutes, blisters are raised on the skin via dermo-epidermal separation through the lamina lucida³³. The procedure is painless. Blister roofs were then removed in a sterile manner and a sterile plastic template placed over the blisters. The template contains 1.0 cm diameter cones that are designed so that one cone fits over each blister base. The cones (~0.8ml volume) were filled with sterile saline alone or with sterile saline containing 10ng/ml human CCL2 (PeproTech, #300-04) and taped to the arm with a pressure bandage to maintain a seal with the skin. One subject did not receive CCL2 in the chamber fluid. The chamber template was removed on the following day at 1000AM and transmigrated leukocytes in chamber fluid were collected and the blister base covered with a sterile dressing. Healing takes place in 5–7 days. Participants served as their own control and received 2 separate treatments immediately after the blisters were created. They first received a control placebo treatment from 1000 to 1600 hours followed by collection of transmigrated leukocytes in chamber fluid 18 hours later on the following day. After a rest period of 3 to 14 days, participants then received a 6-hour cortisol exposure. On these treatment days, blisters were raised on the forearm opposite the one used on the control day. The 6-hour control (placebo) and experimental (cortisol) exposures were deliberately timed so that blisters would be present during the period of GC induced monocytopenia^{34,35} and to coincide with the expected period of peak GC induced inflammatory responses 12 to 24 hours after GC exposure^{4,24}.

2.2 Laboratory protocols

2.2.1 Salivary cortisol measurements—Saliva samples were collected (Salivette, Sarstedt #51.1534) at the time points indicated. Samples were stored at –80°C until analysis by enzyme linked immunosorbent assay (Salimetrics Salivary Cortisol Enzyme Immunoassay Kit, #1-3102, Salimetrics, LLC).

2.2.2 Leukocyte isolation—Heparinized peripheral whole blood samples were layered over Ficoll-Hypaque -1077 (Sigma, #H8889) and centrifuged at 400g for 30 minutes after which mononuclear cells (peripheral blood mononuclear cells, PBMCs) were isolated, washed in saline and counted for subsequent assays. Monocytes were further isolated from mononuclear cells by CD14⁺ bead selection (Miltenyi Biotec # 130-050-201), counted and checked for purity (92–99%).

2.2.3 Peripheral blood leukocyte counts—Peripheral blood leukocytes counts were obtained using a Coulter Counter Z1 (Beckman-Coulter). Differential smears were fixed in methanol and stained with Wright's-Giemsa stain after which 200 leukocytes were counted.

2.2.4 RNA isolation and RT-PCR—Total RNA was isolated from monocytes using the Qiagen RNeasy kit (Qiagen #74104), Qias shredder columns (Qiagen, #79654) and treated with DNase I (Qiagen, #79254). RNA concentration and purity were determined using the RNA6000 Nanodrop. RNA was reverse transcribed using Superscript III First Strand (Invitrogen, #3180808-051). Real-time TaqMan PCR (RT-PCR) was used to quantify mRNA expression using TaqMan Master Mix and validated primer/probe sets (Applied Biosystems). Amplification was carried out using the Applied Biosystems StepOne Real-Time PCR system. Threshold cycle number was determined using Opticon software. mRNA

levels were normalized to β -actin using the formula $2^{-(E_t-R_t)}$ where R_t is the mean cycle threshold for the control gene and E_t is the mean threshold for the experimental gene. Relative fluorescence units were assigned to these values and these data were used to generate the expression profiles delineated in the text. Consistent with our previous studies^{36,37}, no changes in β -actin expression were observed with cortisol treatment excluding this as a source of bias in the analyses. The expression values of experimental genes were therefore normalized to their respective β -actin values. Cycling conditions for RT-PCR consisted of an initial incubation at 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles of 95°C for 15 sec and 60°C for 1 minutes. Product accumulation was measured during the extension phase and all samples were run in triplicate.

2.2.5 Flow cytometry—Recommended volumes (5–20 μ l) of fluorescent labeled antibodies were mixed with PBMNCs in the presence of human γ -globulin (from Cohn fraction II, III; Sigma Chemical, #G-4386) and incubated on ice for 1 hour in dark. Cells were washed once in PBS, pelleted and fixed with 2% paraformaldehyde. Monocyte cell surface markers were evaluated on CD163-positive cells (Trillium CD163-PE, clone Mac-2-158) that were initially gated by forward and side scatter. Data were acquired for fluorescence intensity using a MACSQuant 8 (Miltenyi Biotec). Files were then analyzed using FlowLogic software (Inivai Technologies). UltraComp compensation beads (eBioscience, #01-2222-42) were used as directed by the manufacturer. CD14 (TuK4, Invitrogen); CD163 (Mac-2-159; Trillium); CX3CR1 (2A9-1; MBL Int'l Corp); CCR2 (48607; R&D Systems).

2.3 Statistical analysis

Data are reported as mean $\pm$ S.D. The impact of cortisol exposure on the reported outcomes over time was analyzed using a paired sample t-test with significance achieved at $p < 0.05$. Because of the non-parametric nature of the data, some analyses were completed using the Wilcoxon signed-rank test. Where appropriate, one-way ANOVA for repeated measures was performed to confirm statistical significance.

3. RESULTS

3.1 *In vivo* exposure to stress-associated cortisol concentrations via intravenous infusion increases CCR2 and CX3CR1 mRNA levels and decreases I κ B α mRNA levels in PBMNCs

A preliminary whole genome microarray analysis experiment suggested robust regulation of PBMNC adhesion and chemokine receptor mRNA at selected time points during and after 6-hour *in vivo* exposure to an intravenous infusion of 1.5 μ g/kg/min cortisol. We previously showed that this infusion of cortisol raises plasma and salivary free cortisol to stress-associated levels²⁴. Based on these preliminary results, subsequent participants (n=7) were evaluated by quantitative PCR after receiving the same six-hour intravenous infusion of cortisol described in Section 2.1.1. Peripheral blood drawn before and at 6, 12 and 24 hours after the start of the infusion showed a significant increase in CCR2 and CX3CR1 mRNA levels in PBMNCs. At the same time points, I κ B α mRNA levels showed significant downregulation (Figures 1a–c). Control subjects (n=4), who were not exposed to cortisol, showed no change in these measurements at the same time points. The latter experiments

were completed to examine the potential effect of normal diurnal cortisol variation on these measurements.

3.2 *In vivo* exposure to a stress-associated concentration of cortisol induces transient but significant monocytopenia

As described in Methods, subsequent subjects (n=5) received oral cortisol to avoid the potential confounding effect of intravenous therapy^{38,39}. The cortisol dose led to a mean increase in salivary free cortisol concentration from a baseline of 11.1 $\pm$ 5.0 to a stress-associated concentration of 76.4 $\pm$ 28 nM (Figure 2). We have previously shown this *in vivo* free cortisol concentration to approximate that observed during major systemic stress and to correlate with a plasma cortisol concentration of $\sim$ 40 μ g/dl^{24,32}. Salivary cortisol levels in a separate group of untreated control subjects showed the expected normal diurnal decline during the day. Peripheral blood leukocyte counts were obtained before and at 6, 12 and 24 hours after the start of cortisol exposure. Similar to what has been reported following *in vivo* exposure to high, pharmacologic concentrations of GCs in humans, stress-associated concentrations of cortisol also induced a transient but significant monocytopenia ($0.2 \pm 0.07 \times 10^6$ cells/ml at 6 hours vs. $0.6 \pm 0.2 \times 10^6$ cells/ml at baseline), neutrophilia ($9.3 \pm 3.1 \times 10^6$ cells/ml at 6 hours vs. $4.2 \pm 1.3 \times 10^6$ cells/ml at baseline) and lymphopenia ($0.8 \pm 0.3 \times 10^6$ cells/ml at 6 hours vs. $2.0 \pm 0.8 \times 10^6$ cells/ml at baseline) (Figure 3). Control subjects not exposed to cortisol showed no change in leukocyte counts at the same time points.

3.3 *In vivo* exposure to stress-associated cortisol increases surface expression of CCR2 but not CX3CR1 on CD14⁺ monocytes

As Figure 3 demonstrates, there is a time-dependent alteration in peripheral blood PBMNC leukocyte subpopulations following *in vivo* exposure to stress-associated cortisol concentrations. Consequently, we isolated peripheral monocytes via CD14⁺ selection from the PBMNC population in order to determine stress cortisol effects on the monocyte/macrophage subpopulation. Peripheral blood from participants described above in Section 3.2 was drawn before and at 6, 12 and 24 hours after the start of cortisol exposure and peripheral blood CD14⁺ monocytes were isolated and examined by flow cytometry as described. Monocytes showed a significant increase in expression of CCR2 at the 12 and 24 hour time points (43850 ± 9932 and 36516 ± 6547 S.D. mean MFI, respectively) vs. baseline (24879 ± 6208) ($p < 0.05$ for both). There was no significant change in CX3CR1 expression at these time points (Figure 4a & 4b). Control subjects showed no change in expression of these molecules.

3.4 *In vivo* exposure to stress cortisol increases CCR2 mRNA levels and decreases I κ B α mRNA levels in isolated CD14⁺ cells, but not in lymphocytes

In order to further examine the kinetics of protein regulation we quantified mRNA levels in isolated CD14⁺ monocytes following *in vivo* cortisol exposure. At 6, 12 and 24 hours after the start of a 6-hour *in vivo* exposure to oral cortisol CD14⁺ monocytes and lymphocytes were isolated from peripheral blood mononuclear cells in a different group of participants (n=6). Expression levels of mRNA were measured as described in Methods. As shown in Figures 5a & 5c, cortisol increases CCR2 mRNA at the 12 and 24-hour timepoints, and

decreases IkB α mRNA at the 6 and 12-hour time points in the CD14⁺ cell population (Figure 5a & 5c). CX3CR1 mRNA showed a trend toward increased expression at the 12 hour time point ($p=0.08$) but was not significant (Figure 5b). No changes in mRNA levels were observed in the lymphocyte population at any time point (data not shown).

3.5 Pretreatment with stress cortisol increases *in vivo* monocyte/macrophage and neutrophil migration into a sterile inflammatory site in response to CCL2

Sterile blister chamber fluid in contact with disrupted skin for 24 hours showed a significant increase in the number of macrophages and neutrophils per ml of fluid when subjects were treated with cortisol. These results were significant when blister chamber fluid contained CCL2 but not in the absence of CCL2. Mean macrophage density increased from $3 \pm 1.5 \times 10^6$ to $7.0 \pm 4.7 \times 10^6$ cells per ml ($p<0.05$; Figure 6a) while mean neutrophil density increased from 23 ± 23.6 to 64 ± 34 neutrophils per ml ($p<0.05$; Figure 6b). Although mean macrophage and neutrophil density increased in non-CCL2 containing blister chamber fluid after cortisol exposure, these differences were not significant.

4. DISCUSSION

4.1

Glucocorticoids are best known for their anti-inflammatory effects and for the Addisonian pathology that emerges in their absence. Both of these effects, suppression of inflammation and prevention of Addisonian symptoms, manifest during concurrent *in vivo* exposure to GCs. The experiments presented in this paper are directed towards understanding the recently re-examined adaptive effects of GCs, which are delayed, usually appearing after effector cells are transiently exposed to an increased GC concentration (summarized in Figure 7). Our results show that adaptive, stimulatory effects of GCs on human monocyte/macrophages are measurable, robust and, importantly, are induced by *in vivo* exposure to physiologically relevant GC concentrations. These findings parallel results from animal models, where both enhanced localization⁴⁰ and responsiveness of monocytic cells²³ at the site of an *in vivo* inflammatory stimulus have been observed under similar circumstances.

4.2

Alterations in peripheral leukocyte populations following exposure to high, pharmacologic concentrations of GCs are well-known and include lymphopenia, neutrophilia, and monocytopenia^{34,35,41}. GC-induced lymphopenia was the basis for using GCs in chemotherapy treatments of lymphoid cancer⁴². Neutrophilia from GCs has been ascribed to 'demargination' of neutrophils in the peripheral vasculature⁴¹ and/or inhibition of neutrophil apoptosis for which there is some evidence⁴³. Monocytopenia after GC administration has also been known for some time and was first considered to be a consequence of margination⁴⁴ of monocytes, although direct *in vivo* evidence of this mechanism is lacking. More recent studies in animals provide evidence that the decrease in peripheral blood monocytes observed during GC exposure or stress may be a consequence of enhanced trafficking of monocytes out of the peripheral blood and into tissue sites^{7,15,40,45}. Our results are new and significant because most data in humans have documented changes in peripheral leukocyte populations following exposure to high, pharmacologic concentrations

of GCs in comparison to the physiologic stress-associated concentrations of endogenous GCs investigated in this study. This distinction is important because it indicates a possible physiologic role for endogenous GCs in the development of resistance to external stressors through regulation of leukocyte trafficking. In addition, the effect of cortisol on leukocyte subpopulations was significant despite the *in vivo* variability in cortisol concentrations following administration. Such variability has been previously reported for both intravenous and oral administration of cortisol and appears to be most prominent approximately 6 hours after exposure⁴⁶. Since the bioavailability of oral cortisol is more than 95%, individual differences in cortisol clearance may be the most likely explanation for this observation.

4.3

The data presented here suggest that GC-induced monocytopenia may be mediated through regulation of adhesion and/or chemotaxis molecules. Previous reports have shown GC-induced upregulation of CCR2 on human monocytes *in vitro*⁴⁷ or following exercise-induced increases in circulating cortisol⁴⁸. Notably, monocytes expressing high levels of CCR2 are the monocyte subpopulation that initiates the dynamic process of inflammation and subsequent wound repair^{49,50}. Similar results have been reported for CXCR4 expression and migratory responses of human T lymphocytes⁵¹. In the current study, healthy participants exposed to a stress-associated concentration of cortisol for 6 hours demonstrated a transient but substantial increase in mRNA levels for mediators of leukocyte adhesion and localization. The importance of GC dose merits note, as pharmacological GCs (dexamethasone at 10^{-6} M) have been reported to decrease expression of CX3CR1⁵². During the same period when we found that CCR2 and CX3CR1 mRNA levels increased, mRNA levels of the anti-inflammatory I κ B α protein were significantly decreased. Notably, this downregulation was observed in the absence of a coincident inflammatory stimulus and is therefore consistent with preparatory, pro-inflammatory GC regulation of effector cells. In the absence of external stimulation, the majority of I κ B α is present in the free form unbound to NF κ B, and I κ B α mRNA levels are relatively stable^{53,54}. Conversely, in the presence of an inflammatory stimulus, release of bound I κ B α from NF κ B results in rapid degradation of I κ B α protein and increased I κ B α transcription^{54,55}. Therefore, the GC-mediated decrease in I κ B α mRNA expression prior to inflammatory activation could translate into decreased I κ B α protein levels—i.e. little I κ B α available to bind NF κ B—and an enhanced inflammatory response to a subsequent inflammatory stimulus. We previously reported that pretreatment of human subjects with a 6-hour stress cortisol exposure led to significant increases in circulating levels of IL-6 when subjects were injected with intravenous lipopolysaccharide (LPS) 18 hours later²⁴. This strongly suggests an enhanced NF κ B activation following TLR4 ligation in cells of the monocyte/macrophage lineage, which are a primary target of LPS⁵⁶. Interestingly, and in support of the findings reported here, this study also showed that pretreatment of subjects with stress cortisol was associated with a more significant monocytopenia following *in vivo* LPS exposure compared to control subjects who did not receive stress cortisol²⁴.

4.4

The demonstration that physiological concentrations of *in vivo* GCs enhance CCR2 and CX3CR1 mRNA levels is notable because both of these receptors are associated with

selective migration of pro-inflammatory monocyte/macrophages^{52,57}. The lack of a significant effect on CX3CR1 expression on monocytes is not necessarily evidence against a preparatory role for GCs following stress, since recent work shows that the monocyte subpopulation that initially infiltrates an inflammatory site is CCR2^{hi}CX3CR1^{lo}⁴⁹. Thus, a selective increase in CCR2 expression provides further evidence for the preparative effects of stress-associated GCs on monocytes, which also appear to be the population primarily affected by GC preparatory effects. The observed discrepancy between CX3CR1 mRNA and surface expression could be attributable to a lag in post-translational modification⁵⁸ or because of sequestration, as has been reported to occur when T cells contact activated endothelium⁵⁹. It is also possible that a significant effect on CX3CR1 protein expression would be seen beyond 24 hours, coinciding with the initiation of a resolution phase of inflammation that is predominated by a CCR2^{lo}CX3CR1^{hi} subpopulation of monocytes⁴⁹. Finally, the concept of GC enhancement of inflammatory responses as a consequence of increased cytokine receptor density was introduced into the literature as early as 1992¹⁴ with research since then providing supportive data^{5,8}.

4.5

Although the data presented support our hypothesis that GC-induced monocytopenia is at least partly due to monocyte transmigration following upregulation of adhesion and/or chemotaxis molecules, we interpret this conclusion with caution. Most importantly, peripheral blood leukocyte populations represent a balance between cells leaving the circulation by margination, transmigration, or cell death and cells entering the circulation from precursor or tissue sites. When sampling from peripheral blood, it is rarely clear whether a measured cellular response to a preceding *in vivo* intervention represents alterations in cells pre-existing in the circulation, is a consequence of cells leaving the circulation or of new cells entering the circulation following genotypic or phenotypic modification, or some combination of the above.

4.6

The finding of enhanced macrophage and neutrophil infiltration into an inflammatory tissue site suggests that the above observations have clinical relevance. These cells play a central role in the onset, characteristics and resolution of a tissue inflammatory responses^{49,60}. Neutrophils enter an inflammatory site within minutes followed by an increasing percentage of monocyte/macrophages in tissue over the next 24 hours⁶¹. Although we do not know if the migrated macrophages that we observed in the sterile chamber fluid came directly from the peripheral circulation or whether or they were present in the surrounding cellular matrix, the coincidental finding of monocytopenia suggests that at least some of these cells entered from peripheral blood. Once at an inflammatory site, monocytes differentiate into macrophages that manifest a wide array of regulatory processes to coordinate an ongoing inflammatory process, including its eventual resolution^{49,50}. There is increasing evidence that the latter process is accompanied by ‘activation switching’ in which macrophages convert from a largely pro-inflammatory phenotype to a more anti-inflammatory phenotype^{49,62–65}. Although the inflammatory model we used was limited to measuring cellular infiltration at only one time point, the results suggest that GCs play a role in regulating the magnitude and perhaps the early kinetics of this inflammatory process. An

animal that is stressed, by injury for example, would be primed by the transient release of stress GCs for an enhanced response to a subsequent infection at the initial site of injury, thus demonstrating an adaptive role for GCs.

4.7 Conclusions

In the research described here and in much of the literature cited, an important pattern emerges: transient exposure of leukocyte effector cells to a stress-related concentration of GCs induces a robust increase in the response to a subsequent inflammatory stimulus. The data suggest a 2-step model for GC stimulation of inflammatory responses: First, exposure of effector cells to GCs induces a delayed regulatory response that primes the leukocyte phenotype for an enhanced response to a succeeding inflammatory stimulus. Second, *if* there is a subsequent exposure of the immune cell to an inflammatory signal, enhancement of the inflammatory response is observed. This observation has implications for understanding the role of stress in human inflammatory diseases. For example, does repeated but intermittent exposure to stress predispose some patients to a systemic or localized inflammatory state such as metabolic syndrome, arthritis, colitis or coronary artery disease? It also raises interesting therapeutic questions such as whether preparatory GC effects could be used to enhance responses to bacterial or viral exposures in the settings of elective surgery or vaccination. The ongoing reexamination of GC regulation of inflammation therefore has implications for understanding the role of these ubiquitous hormones in both health and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We transiently exposed healthy humans to stress-associated concentrations of cortisol.
- *In vivo* cortisol exposure increased mRNA levels for adhesion and chemotaxis molecules in isolated peripheral blood monocytes.
- *In vivo* cortisol exposure increased expression of CCR2 on isolated monocytes.
- Cortisol exposure increased the number of macrophages and neutrophils in a sterile inflammatory exudate.
- A model of delayed cortisol augmentation of inflammation is presented.

Figure 1. *In vivo* exposure to intravenous stress cortisol increases mRNA of CCR2 (1a) and CX3CR1 (1b) and decreases mRNA of I κ B α (1c) in PBMNCs (Mean/SEM)

At 6, 12 and 24 hours after the start of a 6-hour intravenous infusion of cortisol (Cortisol), PBMNCs were isolated from peripheral blood as described in Methods. Total RNA was extracted from PBMNCs and CCR2, CX3CR1, and I κ B α mRNA levels were measured using RT-PCR. Values are normalized to β -actin. * = $p < 0.05$; ** = $p < 0.01$ vs. baseline value. A separate group of untreated control subjects were studied and demonstrated that normal diurnal cortisol variations did not affect the same measurements

Figure 2. Salivary free cortisol during stress cortisol exposure and placebo exposure (Mean/SEM) Samples were obtained before (Pre) and at 6, 12 and 24 hours after the start of an *in vivo* exposure of healthy volunteers to either placebo (dashed line) or stress cortisol (solid line). Subjects received oral cortisol administered as a 30mg oral loading dose followed by 10 mg orally every hour for 5 subsequent doses. A separate group of untreated control subjects were studied and demonstrated normal diurnal cortisol variation.

Figure 3. *In vivo* exposure to stress cortisol induces transient but significant monocytopenia (solid line), lymphopenia (dotted line) and neutrophilia (dashed line) (Mean/SEM)

Heparinized peripheral blood samples were obtained before (Pre) and at 6, 12 and 24 hours after the start of an *in vivo* exposure of healthy volunteers to stress cortisol. Subjects received oral cortisol administered as a 30mg oral loading dose followed by 10 mg orally every hour for 5 subsequent doses. **= $p < 0.01$; *= $p < 0.05$ vs. Pre value.

Figure 4. *In vivo* exposure to stress cortisol increases expression of CCR2 (5a) but not CX3CR1 (5b) on CD14⁺ monocytes (Mean/SEM)

Monocytes were isolated (as described in Methods) from peripheral blood at baseline (Pre) and at 6, 12 and 24 hours after the start of a 6-hour exposure to stress associated concentrations of cortisol (Cortisol). Subjects received oral cortisol administered as a 30mg oral loading dose followed by 10 mg orally every hour for 5 subsequent doses. A separate group of untreated control subjects were studied to determine effects of normal diurnal cortisol variation. ** = $p < 0.01$ vs. baseline value.

A

Figure 5. *In vivo* exposure to stress cortisol increases CCR2 mRNA levels and decreases IκBα mRNA levels in CD14⁺ cells (Mean/SEM)

At 6, 12 and 24 hours after the start of a 6-hour *in vivo* exposure to cortisol, monocytes were isolated from peripheral blood as described in Methods. Subjects received oral cortisol administered as a 30mg oral loading dose followed by 10 mg orally every hour for 5 subsequent doses. Total RNA was extracted from monocytes and CCR2 (Figure 5a), CX3CR1 (Figure 5b) and IκBα (Figure 5c) mRNA levels were measured using RT-PCR. Values are normalized to β-actin. * = $p < 0.05$ vs. baseline value.

B

Figure 6. Pretreatment with stress cortisol increases *in vivo* monocyte/macrophage and neutrophil migration into a sterile inflammatory site in healthy human subjects (Mean/SEM)

Participants first received placebo treatment (Control) followed 3 to 14 days later by treatment with cortisol (Cortisol) to raise *in vivo* cortisol concentrations to stress-associated levels. In this way, each participant served as his/her own control. Blister chambers contained either saline or CCL2. Macrophage and neutrophil density in sterile inflammatory blister fluid were assessed 18 hours after each treatment. Mean macrophage (Figure 6a) and neutrophil (Figure 6b) density increased significantly after cortisol treatment in response to CCL2 in the chamber fluid (2.6+/-2.4 SD to 6.7+/-4.7 SD and 40.8+/-42 SD to 63.9+/-34 SD, respectively), but not in the absence of CCL2. * = $p < 0.05$ control vs. cortisol treatment by Wilcoxon signed rank test.

		INFLAMMATORY STIMULUS	
		Absent	Present
CONCURRENENT GC Exposure		Permissive ^{2,11,12}	Anti-inflammatory ^{8,10,14}
PRECEDING GC Elevation		Preparatory ^{21,22}	Pro-Inflammatory ^{4,18,24}

Figure 7. Time and concentration dependent GC effects on *in vivo* cellular responses

Data extracted from both human and animal studies show that *concurrent* exposure to GCs leads to the well-known permissive (anti-Addisonian) effects that sustain normal cellular function in the absence of an inflammatory stimulus. In the presence of an inflammatory stimulus, anti-inflammatory (suppressive) effects are observed when prolonged concurrent elevation of GC levels occurs either in response to the stimulus or through pharmacological intervention. However, a *preceding* and transient exposure to elevated GCs, when followed by a period of low, basal GC concentrations, leads to preparatory effects in the absence of a succeeding stimulus or adaptive effects that are pro-inflammatory (stimulatory) in the presence of a succeeding stimulus.