Full Length Article

Salivary cytokines as a biomarker of social stress in a mock rescue mission

Julia A. Penatzer [a], Julie V. Miller [a], Alice A. Han [a], Nicole Prince [a], Jonathan W. Boyd [b,*]

[a] C. Eugene Bennett Department of Chemistry, West Virginia University, Morgantown, WV, USA
[b] Department of Orthopaedics, West Virginia University School of Medicine, Morgantown, WV, USA

ABSTRACT

Using salivary inflammatory markers as a noninvasive biomonitoring technique within natural social contexts has become increasingly important to link social and biological responses. Many studies have associated circulating cytokines to distinct aspects of physical activity and social/emotional behavior; however, they have not been linked to success and failure in a naturalistic setting for military personnel performing tasks. In this study, salivary cytokines were studied in a group of fifteen Air Force Reserve Officers' Training Corps (ROTC; 14 males, 1 female) subjects performing three mock hostage rescue missions, designed to prompt responses associated with baseline, success, and failure. Each subject completed the tasks of the mission individually and again in randomly assigned teams. Participants were outfitted via direct skin contact with comfortable external Zephyr™ sensors to monitor heart rate, breathing rate, and activity while completing each task. Saliva samples were collected before and after the completion of each mission, and cytokine levels were quantified using enzyme-labelled immunoassay (ELISA) beads. These biomarkers were used to describe the body's immune response to success and failure when performing a mock rescue mission individually and in a team. All measured cytokine levels increased following failed missions performed individually, compared to cytokine levels associated with successful missions. When completing the tasks as a team, there were no significant differences in cytokine response between success and failure; however, being in a team stimulated an increased pre-mission cytokine response, suggesting the concept of teamwork and performing with peers for the first time had a more significant impact than the notion of failing. Additionally, none of the cytokines tested for individual missions correlated to physical activity markers (heart rate, breathing rate, activity) measured during performance. These results indicate a potentially new noninvasive method of determining social stress levels under taxing conditions.

1. Introduction

Social settings are known to stimulate immune responses related to stress and inflammation (La Fratta, Tatangelo, Campagna, et al., 2018; Segerstrom and Miller, 2004; Cohen et al., 2012). In the presence of stress-induced stimuli, the communication between the brain and the body occurs through the autonomic nervous system (ANS), as well as the endocrine and immune systems (McEwen, 2005, 2007). This communication relies on the release of dynamic biochemical messengers to promote adaptation in response to social, mental, and physical stressors (Chida et al., 2008). When these messengers are triggered, they can be measured as early as minutes after an individual perceives the stimuli (Sapolsky et al., 2000).

While measuring these biochemical messengers is critical to understanding the dynamic biological cascades associated with social, mental, and physical stressors, research has been limited because temporal data can be restricted if solely relying on blood as a sample. Sampling with saliva provides a simple, noninvasive method, as an alternative to serum samples, that does not require immediate processing or qualified personnel to collect (La Fratta et al., 2018). Moreover, multiple saliva collections can be obtained on the same day and repeated over time without significant limitations. Saliva consists of 98% water that contains hormones, peptides, electrolytes, mucus, and various enzymes that are also found in the blood (Nunes et al., 2015; Papacoasta and Nassis, 2011). Recent studies have demonstrated blood, the standard diagnostic fluid, is significantly correlated to some salivary biomarkers while monitoring physical activity as well as psychological stress (La Fratta et al., 2018; Nater and Rohleder, 2009; Nunes et al., 2011).

Cytokines are soluble glycoproteins secreted by immune cells and found in both blood and saliva samples (Duque and Descoteaux, 2014; La

Table 1
Summary of subjects.

	Age (years)	Body Mass Index (kg•m^{-2})	Body Fat %
Mean ± SD	19.53 ± 1.1	23.95 ± 2.8	15.52 ± 6.0

Displayed are the means and standard deviation (SD) from the subjects.

Fratta et al., 2018; Prasad et al., 2016). They have been extensively studied in relation to physical activity and short-term exercise, which affects both local and systemic production in response to muscle fatigue (Moldoveanu et al., 2001; Slavish et al., 2015). More importantly, several studies have examined the effects of social stressors on cytokines (Chaing, Eisenberger, Seeman and Taylor, 2012; La Fratta et al., 2018). Dickerson et al. (2004a) demonstrated that individuals who wrote about an experience that induced feelings of shame displayed an increased inflammatory cytokine response versus those who wrote about a control topic (Dickerson, Kemeny, Aziz, Kim, and Fahey, 2004b). Emotional states created by social ties, such as anxiety, anger, and depression, also have been shown to increase proinflammatory cytokines (La Fratta et al., 2018; Dowlati et al., 2010; Denollet et al., 2008). Chiang et al. (2012) demonstrated that individuals who experienced both negative and competitive social interactions displayed heightened proinflammatory cytokines. Additionally, studies have shown social defeat promotes an increase in cytokine response (Audet, Jacobson-Pick, Wann, and Anisman, 2011a; Stanton and Schultheiss, 2010; Wirth et al., 2006). Thus, cytokines provide a new objective and quantifiable biological target that may be explored in relation to success and failure as a response to either physical or social stress.

To explore this concept, Air Force Reserve Officers' Training Corps (ROTC) cadets were enrolled to perform a mock hostage rescue mission individually, and later in teams. Subjects wore an external Zephyr™ (Zephyr Performance System, Boulder, CO) monitor that collected physical activity data during all of the missions. A saliva sample was collected from each subject before the first mission and after the completion of each mission. Cytokine levels were measured using a standard enzyme-labelled immunoassay kit. In contrast to current studies, the present research combines physical activity with salivary cytokine concentrations for individuals in response to both successful and failed missions, while performing as individuals and in teams.

2. Materials and Methods

2.1. Participants

This study received approval from the Institutional Review Board (IRB) committee at West Virginia University (IRB #1511920378) and the United States Army Medical Research and Materiel Command (USAMRMC; IRB #H-24174). Air Force Reserve Officers' Training Corps (ROTC) cadets were enrolled to perform a mock hostage rescue mission. The subjects were introduced to the study during a university ROTC course. A total of 16 subjects volunteered and provided consent for the study; however, one subject failed to provide enough sample to test and was removed from the analysis. Therefore, a homogenous cohort of 15 ROTC cadets (14 males, 1 female) were considered for statistical analyses (Table 1).

2.2. Procedure

Three missions were completed by each individual and, on a separate day, by each team. The subject was given the following mission to read:

"One of the houses outside is a known terrorist stronghold and possible bomb-making facility. After monitoring the facility for some time, we have determined their occupancy habits. At this time, we know the structure to be vacant. It is, however, believed to have a hostage inside; this hostage is believed to know valuable information

and to be sympathetic to our cause. The objective of this mission is to carefully canvass the building, which means leaving no evidence of your presence behind (leave all belongings inside the way you found it, lights off, doors closed), and search each room to find and rescue the hostage (punching bag). It is believed that the hostage has been injured and is possibly unconscious. Our preliminary surveillance has shown that there is a backboard, hand cart, and four bungee cords spread out somewhere in the building. You must find all the items, figure out how to assemble all the items together, and carefully transport the hostage back to the rendezvous point at the garage. I must stress the fact that the building must be left in the state you found it, therefore, move quickly and quietly in the building and use caution when entering and searching each room."

During the first mission, the subjects/teams were instructed to complete the mission while being timed to familiarize the participant with the crime scene house and the task at hand (baseline mission). For the second rescue mission, the subjects/teams were instructed to go as fast as they could, but still abide by the mission objectives and rules (considered a successful mission). During the third hostage rescue mission, the subjects/teams were informed that they would be timed again; however, this time, 1 min into the mission, an air horn was blown and the mission director stated, "Insurgents are returning: You have 1 min left or you will be captured!" The second horn blew 1 min after the first horn and the mission director yelled "FAIL! Assemble the hostage and come back to the rendezvous point" (considered a failed mission). The timer was started for each mission as soon as the subject/team entered the house and ended as soon as they came back to the rendezvous spot and was recorded in seconds. For each mission, all four items (hostage, backboard, hand cart, bungee cords) were randomly assigned to different locations in the house.

2.3. Physical activity markers

Participants were outfitted via direct skin contact with comfortable external Zephyr™ sensors (Zephyr Performance Systems) to continuously monitor heart rate, breathing rate, and activity levels while completing the mission. Heart rate was measured by a sensor in the strap which detects heart electrocardiogram signals, while breathing rate was measured via a pressure pad to detect the expansion of the rib cage, and an internal accelerometer measured the subject activity (Zephyr Technology, 2012). Physical activity markers were calculated using the summed physical activity level (i.e., heart rate) while completing the mission normalized to the time to complete the mission (i.e., beats/second). As previously mentioned, the timer was started as soon as the subject/team entered the house and ended as soon as the subject(s) came back to the rendezvous spot.

2.4. Saliva samples

Saliva samples were collected before the first mission (pre-mission; approximately 19:00) and after the completion of each mission (baseline, success, and failure). Prior to saliva collection, subjects were given an 8 oz bottle of water and instructed to immediately drink it. Ten minutes after each subject finished their water, a 1-mL sample was taken with the help of a saliva collection aid (Salimetrics, Carlsbad, CA). Immediately after collection, the saliva samples were stored on ice and later transferred to a freezer (−80 °C) until analysis. On the day of analysis, samples were centrifuged at 3000 rpm for 15 min at 4 °C.

2.5. Cytokine 10-plex assay

The saliva supernatant was assayed for the following cytokines via an ultrasensitive human cytokine 10-plex panel including: interleukins (ILs)- 1β, 2, 4, 5, 6, 8 and 10, granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor alpha (TNF-α), and interferon

gamma (IFN-γ) (Fisher Scientific, Pittsburgh, PA). The samples were analyzed with the Bio-Plex 200 suspension array system and Pro II Wash Station (Bio-Rad, Hercules, CA), according to the manufacturer's instructions and performed in triplicate (inter assay variation <10%).

2.6. Statistical analysis

All data analyses were performed using GraphPad Prism V5 (San Diego, CA) and SAS JMP Pro V14 (Cary, NC). The samples were quantified using the provided standards from the kit. Two cytokine targets, IL-1β and IL-8, were consistently above the highest standard for all participants and were removed from the dataset. Responses from the female subject did not significantly affect the analyses and therefore was included in all of the datasets. Data are presented as means ± standard error of the mean (SEM), and significance tests were performed using the Friedman test or the Wilcoxon matched-pairs signed rank test, where appropriate. Dunn's post-test was also performed to determine significant changes in cytokine levels across missions. Values were considered statistically significant at a 5% level of significance ($p < 0.05$). Pearson correlation analyses were also conducted for cytokine and physical activity marker data; however, none of the correlations were considered statistically significant at a 5% level of significance ($p > 0.05$) (data not shown). Additionally, Pearson correlations were run between cytokine concentrations and age, body fat percentage, and body mass index (BMI) for each subject to determine the possible contribution of these variables. None of the correlations between cytokine levels and age, body fat percentage, or BMI were significant ($p > 0.05$) (data not shown).
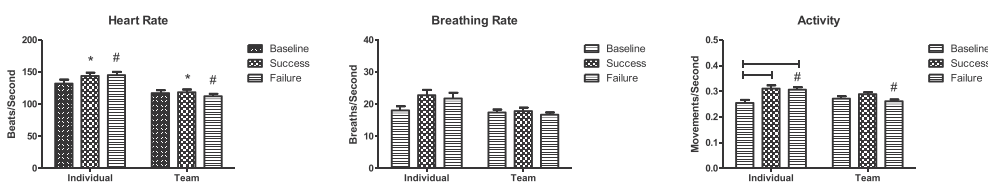
3. Results

3.1. Physical activity markers of stress

Using external Zephyr™ sensors, the physical activity markers (heart rate, breathing rate, and activity) were calculated using the summed marker normalized by the time (seconds) it took to complete the mission. There was a significant increase in activity for individuals compared to baseline for success and failure ($p < 0.05$). Conversely, there was no significant difference for success and failure for individuals or for teams for heart rate, breathing rate, and activity ($p > 0.05$) (Fig. 1). However, heart rate and activity were significantly decreased for individuals in a team compared to individual tasks for failed missions. Heart rate was also significantly decreased for individuals in a team for successful missions.

3.2. Individual salivary cytokine response to success and failure

Cytokine concentrations were measured in saliva when the subjects first arrived (pre-mission) and 10 min post completion of each mission (baseline, success, and failure). All eight cytokines displayed significant increases ($p < 0.05$) following the failed mission compared to success

(Fig. 2) determined by the Friedman test with Dunn's post-test. Conversely, a significant decrease in cytokine response was observed following the successful mission compared to baseline for IL-6 and TNF-α (Fig. 2).

3.3. Team salivary cytokine response to success and failure

Cytokine concentrations were also measured in saliva after the team missions. While performing as a team, none of the cytokines displayed significant differences between baseline, success, and failure (Fig. 3). However, the baseline cytokine response for individuals on the day of the team mission were significantly increased ($p < 0.05$) compared to the day of individual completion determined by Wilcoxon matched-pairs signed ranked test (Fig. 4).
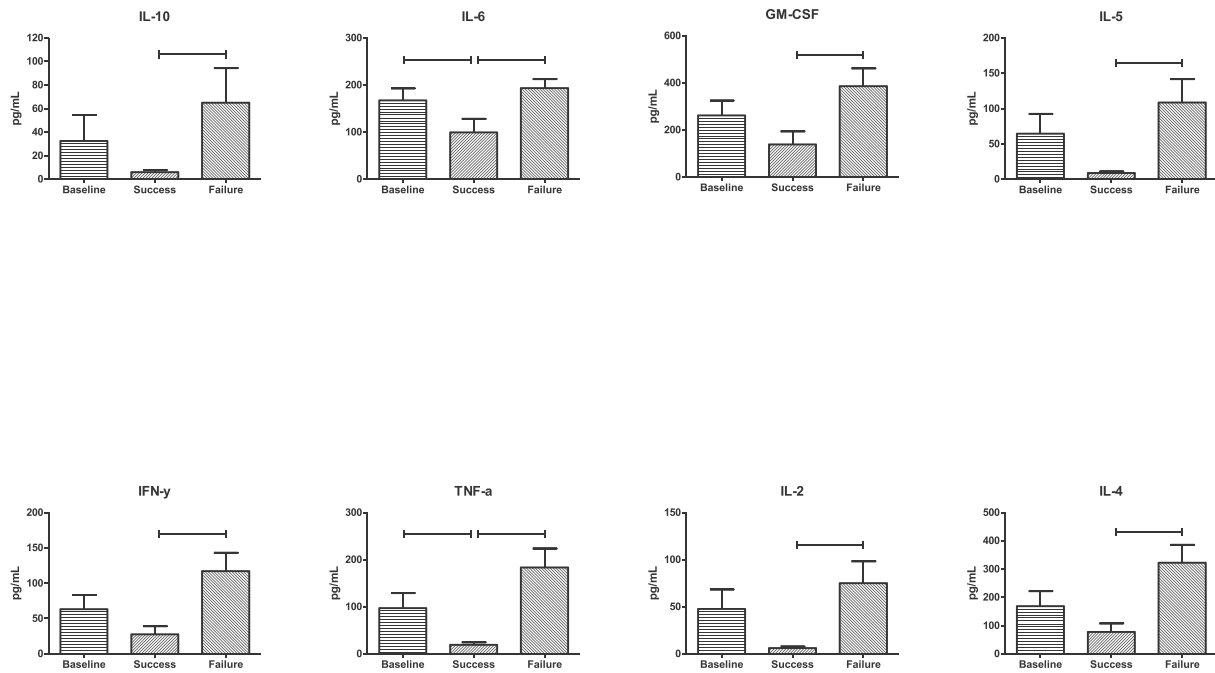
4. Discussion

Social ties are highly important for survival, and thus threats to our social connections (e.g., rejection, isolation, conflict, or loss) signal to our body that one is more vulnerable and may face a greater likelihood of wounding and infection and, as such, a greater need for inflammatory activity marked by increased cytokine concentrations is needed (Eisenberger et al., 2017). Within this study, Air Force ROTC cadets were enrolled to perform a mock hostage rescue mission and cytokine responses were measured in combination with physical activity to better understand the individual stress response to success and failure in high stress field situations.

The Zephyr™ bioharness device provides reliable and valid measurements that can be continuously taken and monitored while completing activities (Nazari et al., 2018). The wearable device provides various physical activity parameters, such as heart rate, breathing rate, and activity levels, which are important in monitoring physical intensity during exercises. Within our study, there were no significant differences in physical load as determined by heart rate, breathing rate, and activity levels between success and failure while completing the missions as individuals or in a team setting. However, we did observe an increased response for individuals for success and failure from baseline for activity/time. Due to the fact that participants were instructed to complete tasks as quickly as possible during the successful and failed missions, it was expected that the subjects would move more efficiently during these two tasks. Additionally, there was a decreased response for the averaged team heart rate and activity from the individual metrics, which is expected due to the shared physical load while completing the mission in teams.

Salivary biomarker analysis provides a unique opportunity to collect saliva multiple times on the same day, without significant limitations. As such, within this study, cytokine levels were able to be monitored following completion of each mission. After a failed mission, cytokine levels were found to be significantly increased for all of the measured

Fig. 1. Physical activity markers of stress were measured for individuals (n = 15) while completing a mission as individuals and part of a team using external Zephyr™ sensors. Physical activity markers were calculated using the summed activity (generated by Zephyr™) normalized by the time (seconds) it took to complete the mission as described in Materials and Methods. The physical activity metric was averaged for all individuals. Significance levels were determined using the Friedman test with a Dunn's post-test, where # and * indicate significance ($p < 0.05$) between an individual and team mission. Bars indicate significance ($p < 0.05$) within individual or team missions. Data are represented as mean ± SEM.
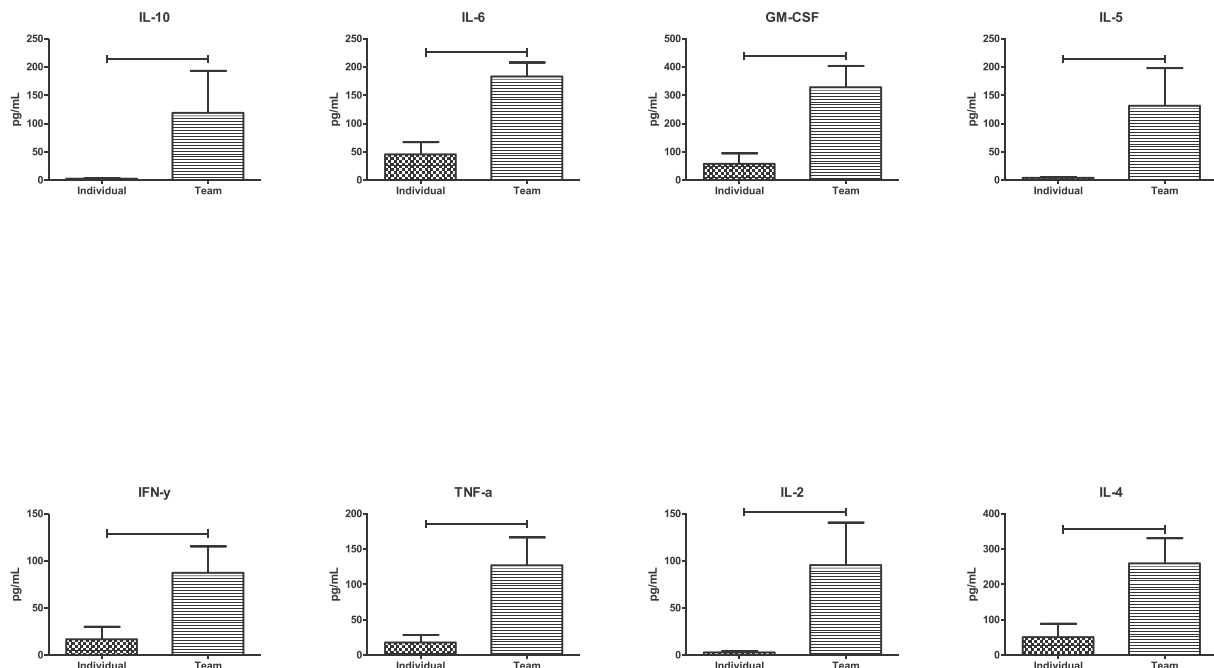
Individual

Fig. 2. Salivary cytokine concentrations were measured for individuals (n = 15) while completing a mission as individuals. Cytokine responses (IL-10, IL-6, GM-CSF, IL-5, IFN-g, TNF-a, IL-2, IL-4) were measured in saliva after each mission using a multiplex ELISA as described in Materials and Methods. Significance levels were determined using the Friedman test with a Dunn's post-test. Bars indicate significant differences (p < 0.05) between missions. Data are represented as mean ± SEM.

Team
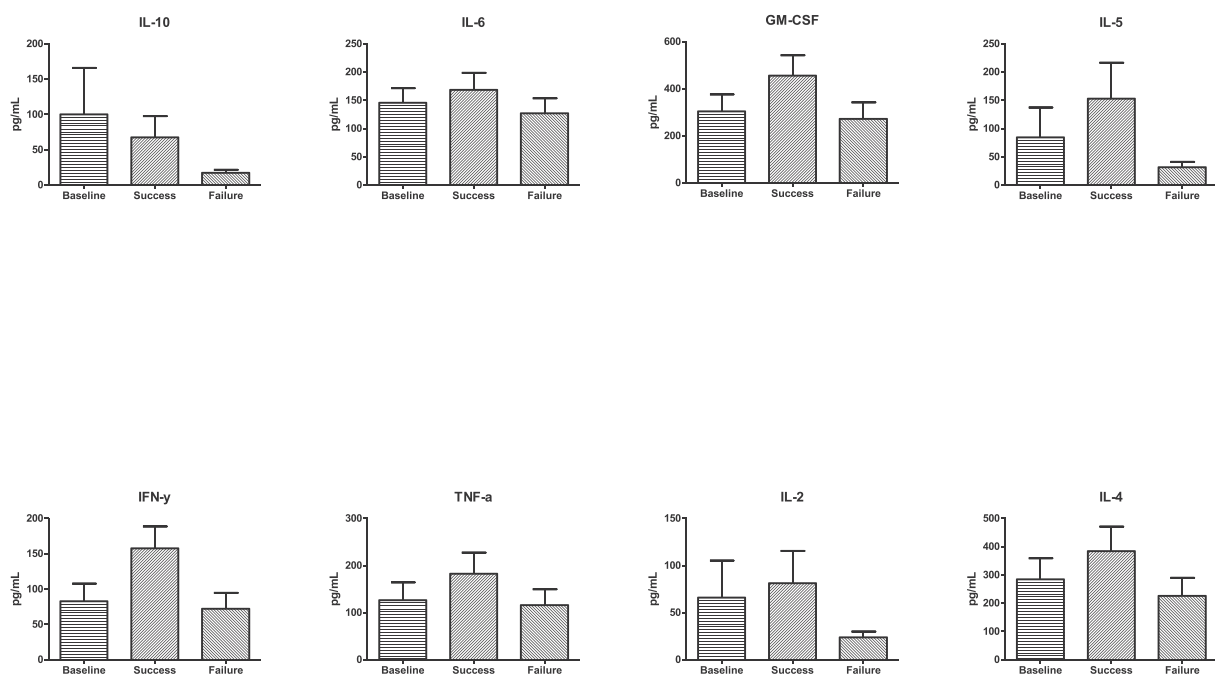
Fig. 3. Salivary cytokine concentrations were measured for individuals (n = 15) while completing a mission on teams. Cytokine responses (IL-10, IL-6, GM-CSF, IL-5, IFN-g, TNF-a, IL-2, IL-4) were again measured in saliva after each mission. There were no significant differences (p > 0.05) as determined by the Friedman test with a Dunn's post-test. Data are represented as mean ± SEM.

Pre-Mission

Fig. 4. Cytokine responses (IL-10, IL-6, GM-CSF, IL-5, IFN-g, TNF-a, IL-2, IL-4) were also measured for individuals (n = 15) before completing the missions as individuals and as teams. Significance levels were determined using the Friedman test with a Dunn's post-test, where bars indicate significant differences (p < 0.05) between individual and team baseline. Data are represented as mean ± SEM.

cytokines compared to success (Fig. 2). These increased responses of failure may be reflective of a defeat resulting in psychological stress or anxiety (Audet, Mangano, Anisman, 2011b; Takahashi et al., 2018). In addition to the 2004 study, Dickerson et al. (2009) demonstrated that individuals who performed the Trier Social Stress Test in front of an evaluative panel showed increases in proinflammatory cytokines from pre-to post-stress compared to those individuals who performed the same task, but completed it alone (eliminating the social stress). Likewise, in our experimental setup, the individuals returned to the rendezvous point where all the investigators and ROTC commanders were waiting. Thus, these increased cytokines may be in response to the social defeat experienced in front of a group. Conversely, successful missions resulted in decreased cytokine levels compared to baseline for IL-6 and TNF-α (Fig. 2). This lack of inflammatory response is likely a feeling of relief or acceptance within the social setting after performing well.

Pearson correlation analyses were completed to determine if any significant correlations existed between physical activity metrics and cytokine levels within each mission. There were no significant correlations (data not shown) for any of the individual missions to the physical activity data. These results indicate the cytokine responses observed for individual failure and success for the subjects undergoing a mock rescue mission were independent of physical stress.

To further understand if the cytokine responses were a result of social or mental stress, a repeat study was performed on the same individuals in a team environment. Neither success nor failure caused a significant difference while performing as a team (Fig. 3). Thus, in the team setting, the same response to success and failure is not experienced, but they were still experiencing the same mental stimulation. These results indicate that the increased inflammatory response is not a result of mental stress.

Interestingly, the pre-mission cytokine levels measured on the day of completion for individuals in a team were significantly higher than on the day of individual mission (Fig. 4). The human body, especially the endocrine, immune, and cardiovascular systems, is exceedingly sensitive and responsive to social interactions (Heaphy and Dutton, 2008). In this study, cytokine responses were immediately heightened in a team setting

compared to an individual setting without any physical or mental stimulation. Thus, the increased immune response to the team setting further indicates the elevated cytokine levels in response to failure for individuals are in response to social stressors.

In summary, using a noninvasive biomonitoring technique, this study was able to determine cytokine levels in response to success and failure. Furthermore, individuals experienced higher cytokines in response to failure and to a team setting. Additionally, the individual cytokine responses did not correlate to physical load as measured by a Zephyr™ bioharness. These cytokines could be important to better understand social stress during a physical exercise, especially for military personnel (and possibly could be extended to sports teams in the future). Importantly, this study was conducted in a naturalistic setting for these personnel and followed subjects through three distinct scenarios (baseline, success, and failure). It should be noted that one limitation of this study was small sample size (15 subjects). However, the sample size does meet statistical power analysis standards, and future research will aim to increase the size of the teams to explore larger groups. While these results indicate a noninvasive method of determining social stress levels under challenging conditions, this relationship should be further investigated to better understand potential differences in cytokine levels among genders.

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