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Title: Acute psychological stress induces short-term variable immune response

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Abstract

In spite of advances in understanding the cross-talk between the peripheral immune system and the brain, the molecular mechanisms underlying the rapid adaptation of the immune system to an acute psychological stressor remain largely unknown. Conventional approaches to classify molecular factors mediating these responses have targeted relatively few biological measurements or explored cross-sectional study designs, and therefore have restricted characterization of stress-immune interactions. This exploratory study analyzed transcriptional profiles and flow cytometric data of peripheral blood leukocytes with physiological (endocrine, autonomic) measurements collected throughout the sequence of events leading up to, during, and after short-term exposure to physical danger in humans. Immediate immunomodulation to acute psychological stress was defined as a short-term selective up-regulation of Natural Killer (NK) cell-associated cytotoxic and IL-12 mediated signaling genes that correlated with increased cortisol, catecholamines and NK cells into the periphery. In parallel, we observed down-regulation of innate immune toll-like receptor genes and genes of the MyD88-dependent signaling pathway. Focusing analyses on discrete groups of coordinately expressed genes (modules) throughout the time-series revealed immune stress responses in modules associated to immune/defense response, response to wounding, cytokine production, TCR signaling and NK cell cytotoxicity which differed between males and females. These results offer a spring-board for future research towards improved treatment of stress-related disease including the impact of stress on cardiovascular and autoimmune disorders, and identifies an immune mechanism by which vulnerabilities to these diseases may be gender-specific.

1. Introduction

Chronic psychosocial and emotional distress impact immune function which leads to increased risk for disease. Current estimates forecast that by year 2030, stress-related pathologies will lead as the most debilitating and widespread health disorders (Mathers et al., 2008). At the same time, while chronic stress-related effects upon the immune system are uniformly deleterious, acute stress appears to have both protective and adverse effects. For example, acute stress can enhance the acquisition and expression of immunoprotection by activation of bodily defences prior to wounding or infection (Ackerman et al., 2002; Amkraut et al., 1971; Carney, 2004; Dhabhar, 2009), or alternatively induce immunopathology via exacerbating autoimmune inflammation, with respiratory and cardiovascular consequences (Al'Abadie et al, 1994; Black, 2006; Bosch et al., 2003; Dhabhar et al., 1995; Garg et al., 2001). The dissociation between excitatory and inhibitory molecular mechanisms remains incomplete. A more detailed understanding of immunomodulation throughout acute stress in humans is necessary not only to clinically reduce immunopathology, but also to harness stress-related immunoprotective effects.

One primary mechanism by which acute psychological stress induces immune response is through rapid changes in leukocyte distributions in the peripheral circulation (Bosch et al., 2008). Studies investigating acute short-term stressors in humans, such as public speaking, have reported brief increases of natural killer (NK) cell numbers and other leukocyte subtype cell numbers, a reduction in lymphocyte proliferation, an increase in pro-inflammatory cytokine production, and reduced healing capacity of the skin (Altemus et al., 2001; Segerstrom and Miller, 2004). Studies of acute (psychological) stress due to physical danger have used first-time tandem skydive (Schedlowski et al., 1993), as this challenge has the advantage of representing real risk, eliciting reliable effects, and yet permitting a high degree of experimental control. Studies using this paradigm report transient increases of T cells and NK cells in the blood, as well as a parallel increase in NK cell cytotoxic activity. This suggests that

changes in leukocyte numbers may be an important mediator of apparent changes in leukocyte activity. Comparably, an equivalent study of bungee jumping reported increases in neutrophils, pro-inflammatory monocytes, and $CD8^+$ T cell numbers following the jump (van Westerloo et al., 2001).

While these studies are suggestive, one important limitation until recently has been the lack of computational and molecular approaches for large-scale immune system monitoring. Microarray analysis of blood transcriptional profiles offers a means to investigate immunological mechanisms relevant to acute psychological stress on a genome-wide scale. To complement these data, network analyses have been used in the field of immunology to identify the groups of coordinately expressed transcripts (modules) that are involved in the response of immune cells to immunomodulatory factors (i.e. acute stress). Indeed, the probability for multiple transcripts to follow a complex pattern of expression across dozens of participants throughout a time-series only by chance is low, and such sets of genes should therefore constitute coherent biologically meaningful transcriptional modules.

To exploit these capabilities, we performed a detailed molecular and cellular analysis upon two cohorts of participants undergoing their first-time tandem skydives. We first applied a comparative analysis of peripheral blood leukocyte (PBL) gene expression profiles between the four time-points (i) baseline, (ii) leading up to, (iii) during, and (iv) after each skydive to identify a unique panel of candidate stress responsive genes, which were validated by RT-qPCR assays. An unsupervised network analysis was then used to identify coordinately expressed genes (modules) involved in the short-term variable immune response to acute stress while considering gender-specific effects. Finally, the implications of gene expression analysis with respect to cell subset changes were validated by flow cytometry on a second cohort of participants.

2. Materials and Methods

2.1 Ethical approval

State University of New York at Stony Brook and the University of California San Diego Institutional Review Boards approved this study. Thirty-nine skydivers participated in this study consisting of 13 subjects for RNA expression profiles (7 male, 6 female) and 26 subjects for flow cytometry (17 male, 9 female). All skydivers provided written consent prior to participation. Participants were recruited from individuals who independently contacted an area skydiving school (Skydive Long Island, Calverton, NY) to schedule their first-time tandem skydive. Skydivers were healthy adult subjects with no history of cardiac or mental illness, as determined by physical examination, medical history, and screening using the Structured Clinical Interview for DSM-IV.

2.2 Subjects and sample collection schedule

The study protocol adhered to a strict timeline for sample and data collection. Baseline blood samples were collected at 9:15 am within one week prior to or after the day of the skydive during a hospitalized testing that was time-locked to data collection during the skydive day and therefore served as a baseline and control. On the skydive day, all skydivers awoke at 6:30 am and arrived at Stony Brook University Hospital at 7:30 am. “Pre-boarding” samples were collected at 9:15 am, one hour before take-off. Take-off occurred at 10:15 am, and the jump occurred at 10:30 am when the airplane reached an altitude of 11,500 feet (3,505.2 meters). Skydivers landed at about 10:35 am and “post-landing” samples were collected at 10:45 am. Skydivers were immediately transported to Stony Brook University Hospital for a final blood draw at 11:30 am (“one hour post-landing” sample). Saliva was collected every 15 minutes from 9:15 am to 11:30 am on both the skydive and baseline hospital day.

2.3 RNA isolation and microarray gene expression analysis

Ten milliliters of blood were collected for each blood draw in an EDTA coated vacutainer blood collection tube and leukocytes were fractionated by passing the blood through LeukoLOCK filters. RNA isolation was performed using the LeukoLOCK Total RNA Isolation Kit and 100ng of total RNA were used as starting material. RNA with a 260/280 ratio > 1.7 and a RIN > 6 was

considered suitable for microarray analysis. Synthesis of cDNA and biotinylated cRNA and hybridization of cRNA to Illumina HumanHT12 v4 BeadChips (47,231 probes). Because the integrity of RNA was of low quality for three subjects, partially paired data was analyzed (**Table S1**).

2.4 Data pre-processing

Quality control of microarray data, variance-stabilizing transformation (vst), robust-spline normalization and removal of genes not expressed in any of the samples was performed in the R statistical computing environment version 2.8.0, using the Bioconductor package *lumi* (Du et al., 2008). Probes lacking gene symbol annotations were removed while probes with duplicate gene symbols were selected on the basis of having a higher average expression across all samples. This final filtering step left a total of 18,129 probes that passed into our subsequent analyses. We used two methods to identify outlier samples (2.5 standard deviations $\pm$ mean) for quality control: clustering analysis based on Pearson correlation and average distance metric and principal component analysis (PCA) using the first three components. This reduced our sample size from 50 subjects to a total 45 subjects (**Table S1**). The resulting quality-control treated data were used as input for differential expression and WGCNA analyses.

2.5 Differential gene expression analysis

We measured differential expression with respect to gene expression at baseline for each time point using 18,129 probes, correcting for gender differences. Differentially expressed genes were assessed using the moderated t-test in LIMMA (Smyth, 2005), and unless otherwise specified, a highly statistically significant threshold of p-value < 0.01 was used. To ensure that genes found significantly differentially expressed post-landing were not solely a consequence of increased proportion of NK cells, we used a multivariate linear model to regress individual gene expression levels against NK-cell specific marker genes. The criteria for classification as a NK-cell marker were that the genes must be these particular genes needed to be: 1) identified in multiple publications linking them to the NK-cell type; and 2) found intersecting

across three independent cell type specific expression databases [CTen (Shoemaker et al., 2010), IRIS (Abbas et al., 2005), and HaemAtlas (Watkins et al., 2009)]. Like others whom have made similar corrections (Miller et al., 2013), we note that the model is fairly robust to choice of marker genes for cell type.

2.6 Weighted gene co-expression network analysis and module characterization

The process of identifying discrete groups of co-regulated genes can be divided into two steps. First, a signed global co-expression network was built with weighted gene co-expression network analysis (WGCNA) in R using normalized expression data of 18,129 probes. For each set of probes, a pair-wise correlation matrix was computed using the Pearson correlation. WGCNA weights the Pearson 'correlation matrix' by taking their absolute value and raising them to the power ß, producing an 'adjacency matrix' (Langfelder and Horvath, 2008). This step emphasizes strong correlations and punishes weak correlations on an exponential scale. We only consider those powers that lead to a network satisfying scale-free topology at least approximately ($R^2 > 0.80$) so the mean connectivity is high and the network contains enough information (e.g. for module detection). We found that our microarray data needed a ß of 9 to reach a scale-free fit. Second, the adjacency matrix was used to calculate the topological overlap measure (TOM), representing the overlap in shared neighbors. The dissimilarity TOM was used as input for the gene dendrogram (i.e. gene tree of closest pairwise neighbors), and co-expression modules were detected as branches of the gene dendrogram using the hybrid tree-cut algorithm (**Fig. S4**) (Langfelder and Horvath, 2008). With minimal module size set to 15 probes and merging threshold set to 0.1, 20 modules were detected.

To integrate physiological measurements with these co-expression modules, we ran singular value decomposition of each module's expression matrix and used the resulting module eigengene (ME), equivalent to the first principal component, to represent the overall expression profiles for each module. Subsequently, MEs for all modules were correlated to recorded clinical and physiological parameters such as nerve growth factor, epinephrine, norepinephrine, beta endorphin, heart rate, state anxiety trait and cortisol levels which provide

a complementary assessment of these potential confounders to that performed in standard differential expression analysis. MEs are also useful for decreasing the amount of sample space tested in terms of reducing the number of multiple comparisons. A Bayes ANOVA (parameters: conf=12, bayes=1, winSize=5) (Kayala and Baldi, 2012) was used to compare ME expression values for modules of interest across time-points while taking into account gender differences. For each gene in a module, intramodular membership (kME) was defined as the correlation between gene expression values and ME expression. Genes with high kME inside co-expression modules are labeled as hub genes and are predicted to be of essential to the function of the module.

2.7 Gene enrichment analyses

All differentially expressed genes passing a p-value < 0.01 and all 20 network modules with genes passing a kME > 0.50 were subjected to functional annotation. First, the ToppFunn module of ToppGene Suite software (Division of Biomedical informatics) (Chen et al., 2009) was used to assess enrichment of GO ontology terms associated to relevant biological processes and pathways based on a one-tailed hyper geometric distribution with a Bonferroni correction. All annotations must have contained at least two genes to be allowed for testing. Second, to predict the involvement of key cell types we utilized the cell specific (HECS) gene expression database from the cell type enrichment (CTen) analysis web-based tool compiled by (Shoemaker et al., 2011) for a broad characterization of cell type specific expression. For each gene list supplied, the significance of cell type specific expression is determined using the one-tailed hyper-geometric distribution with a Bonferroni correction across all cell/tissue types.

2.8 Protein interaction networks

Protein-protein and protein-DNA interactions for products of differentially expressed genes at pre-boarding, post-landing and one hour post-landing were determined using the direct interactions algorithm in MetaCore™ (GeneGo, St. Joseph, MI). The interactions documented

in MetaCore™ have been manually curated and are supported by citations in the literature record. When protein networks are constructed, they often reveal hub genes which represent transcription factors that control the regulation of multiple target genes. Visualization of a direct protein interaction network was facilitated by use of Cytoscape (Shannon et al., 2003).

2.9 Real time RT-q PCR

Twenty-two targets were chosen for RT-qPCR confirmation of gene expression. To rule out false positives, 15 components of NK cell-mediated cytotoxicity pathway and 3 transcription factors were selected: killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1 (*KIR3DL1*), killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 1 and 4 (*KIR2DL1* and *4*), killer cell lectin-like receptor subfamily D, member 1 (*KLRD1*), killer cell lectin-like receptor subfamily C, member 2 (*KLRC2*), natural cytotoxicity triggering receptor 3 (*NCR3*), Fas ligand (*FASLG*), perforin 1 (*PRF1*), granzyme B (*GZMB*), lymphocyte-specific protein tyrosine kinase (*LCK*), zeta-chain (*TCR*) associated protein kinase 70kDa (*ZAP70*), linker for activation of T cells (*LAT*), SH2 domain containing 1B (*SH2D1B*), interferon gamma (*IFNG*), CD247 molecule (*CD247*), runt-related transcription factor 3 (*RUNX3*), FBJ murine osteosarcoma viral oncogene homolog (*FOS*), interferon regulatory factor 1 (*IRF1*). To rule out false negatives, 3 targets were selected: killer cell lectin-like receptor subfamily K, member 1 (*KLRK1*), cathepsin C (*CTSC*) and transcription factor T-box 21(*TBX21*, also known as T-bet). One gene not detected by microarray was selected to test possibility of the presence of faulty probes – natural cytotoxicity triggering receptor 1 (*NCR1*). When available, TaqMan® Gene Expression Assays (Applied Biosystems by Life Technologies, Carlsbad, CA) were selected that matched the region of the RNA targeted by the corresponding Illumina probe as closely as possible; otherwise, custom assays were designed and ordered from Integrated DNA Technologies, Inc. (Corallville, IA). Reverse transcription reactions were performed using qScript™ cDNA SuperMix (Quanta Biosciences, Inc., Gaithersburg, MD). *GAPDH* control assay was used as a normalizer. Fold changes were obtained using DataAssist software version 3.01 (Applied Biosystems by Life Technologies, Carlsbad, CA) using the $2^{-\bullet\bullet CT}$ method. To determine significance, a paired *t*-test or Wilcoxon test (depending on the normality of the distribution as assessed by Shapiro test) was

performed using normalized *Ct* values (target Ct - *GAPDH* Ct) between the time point of interest and baseline samples. Genes with *p*-values < 0.05 were considered significant.

2.10 Flow cytometry

Two blood samples were collected from an additional cohort of 26 first-time tandem skydivers for flow cytometry analysis (one for complete blood counts and a second tube for flow cytometry data analysis). Aliquots from each blood sample were placed into 8 tubes (panels) and incubated with the mAb combinations using the manufacture's recommended procedures. After incubation, sample processing for the flow cytometry analysis followed the manufacture's instruction using red blood cell (RBC's) lysing solution (Becton Dickinson, San Jose, CA). After lysing the RBC's, the white blood cells were washed in phosphate buffered saline (PBS) and re-suspended in PBS buffer and analyzed using a FACS Caliber 4-color flow cytometer (Becton Dickinson, San Jose, CA). Expression of cell-surface proteins labeled with R-Phycoerythin (PE) was quantified using the geometric means of the mean florescence intensity (MFI) (Shapiro, 2003). All mAb's were purchased from BD Biosciences Pharmingen (San Diego, CA).

3. Results

In this exploratory study, we induced 'real-world' acute psychological stress in response to a first-time tandem skydive. Subjects reached altitude in fifteen minutes, jumped at 13,000 feet (4km), fell at terminal velocity for one minute, and parachuted for another four minutes prior to landing. PBL samples and circulating hormone measurements from thirteen participants (7 male and 6 female) were collected at baseline (9:15 am one week before/after the skydive day), pre-boarding (9:15 a.m. skydive day), post-landing (10:45 a.m. skydive day, immediately after landing) and one hour post-landing (11:45 a.m. skydive day) (**Fig. 1A**).

3.1 Fluctuations in endocrine and autonomic measurements in response to acute stress

Testosterone, norepinephrine, epinephrine, beta-endorphin, nerve growth factor (NGF), salivary cortisol and heart rate were monitored throughout both the baseline and skydive days as well-established biomarkers for HPA-axis activation consequent to acute psychological stress. Heart rates were elevated on the skydive day relative to baseline as early as pre-boarding the airplane (09:45-09:55) and remained elevated until 30 minutes post-landing (10:30-11:00), peaking immediately before existing the airplane (10:25-10:30, p=6.04E-05) (**Fig. 1B**). Salivary cortisol measurements were taken every 15 minutes, starting pre-boarding (09:15) to one hour post-landing (11:35) at both the baseline and skydive days. On the skydive day, a significant increase in salivary cortisol was observed immediately before exiting the plane (10:15, p=8.0E-03) and peaked between jumping and one hour post-landing (10:30 p=5.0E-04; 10:45, p=5.0E-03; 11:00, p=2.0E-02) (**Fig. 1C**) compared to the same time-points at baseline. A moderate, yet insignificant, increase of circulating testosterone, beta-endorphin and NFG was observed from baseline to post-landing **(Table S1)**. Circulating levels of norepinephrine and epinephrine increased post-landing relative to baseline (p=4.0E-02, p=3.0E-02) (**Fig. 1D-E**). Heart rate, salivary cortisol and catecholamine levels returned to baseline levels one-hour post-landing. These patterns support stress-induced HPA activation that occurred in response to the stress of skydive. Therefore, gene expression signatures that closely followed changes in these physiological responses were expected.

3.2 Identification of candidate acute stress responsive genes

To identify stress response genes that were non-gender specific, PBL gene expression profiles were corrected for gender differences at pre-boarding, post-landing and one-hour post-landing relative to baseline. Differentially expressed genes (all $p < 0.01$) were identified pre-boarding (N=94), post-landing (N=373) and one-hour post-landing (N=121) relative to baseline (**Fig. 2 A-B;** for lists of differentially expressed genes see **Table S2**). The majority of gene expression differences were detected at post-landing and visualized on a heatmap to compare expression levels of these genes at other time-

points (**Fig. 2C**). Genes modulated pre-boarding and one-hour post-landing displayed no functional characteristics or leukocyte cell type specificity. However, of the 373 differentially expressed genes identified from baseline to post-landing, NK cell cytotoxicity and IL-12 signaling genes, including IFN- , were up-regulated (**Fig. 2D**). Genes related to MyD88-dependent toll-like receptor (TLR) signaling tended to show decreased expression. Additionally, cell type enrichment analysis revealed a significant enrichment of up-regulated genes post-landing specific to $CD56^{+}$ NK cells, and to a lesser extent $CD8^{+}$ T cells (**Fig. S3A**).

Key genes, including those encoding transcription factors, involved in mediating stress-immune interactions were discovered through interactome analysis of all differentially expressed genes, utilizing validated direct protein-protein interaction (PPI) information from MetaCore™ (**Fig. S1**). This analysis revealed the up-regulation of transcription factors *RUNX3*, *FOS*, *JUN* of the innate immune system and cyclin-dependent kinase inhibitor 1A (*CDKN1A)* and zeta-chain (TCR) associated protein kinase 70kDa (*ZAP70)* of the acquired immune system. Mitogen-activated protein kinase 3 (*MAPK3*), malic enzyme 2 (*ME2*) and guanine nucleotide binding protein (*GNAI*) mediating innate immune events were down-regulated.

3.3 RT-q PCR validation of selective NK cell cytoxocity response

A set of independent RT-qPCR assays were used to verify differentially expressed genes (from microarray data) post-landing. The RT-qPCR analysis was conducted on 22 of the differentially expressed genes that play a key role in the NK cell cytotoxicity response (**Fig. S2**). These genes include those that encode inhibitory receptors (*KIR2DL1*, *KIR3DL1)* and activating receptors (*KIR2DL4*, *KLRC2*, *KLRD1*, *NCR3*), classical MHC class 1 molecules (*HLA-C, B, E, G*) which bind to the receptors, adapter molecules for activating receptors (*SH2D1B, CD247*), signal transduction molecules (*LAT, LCK, ZAP70*) important for NK and T cell activation, cytolytic granules (*PRF1,*

GZMB), and transcription factors (*RUNX3, FOS*). Based on previous reports of NK cell mobilization into blood in response to acute stress, it was probable that a significant number of genes would map to NK cell mediated cytotoxicity pathway (Altemus et al., 2001; Schedlowski et al., 1993). However, not all well characterized NK cell related molecules, pro- and anti-inflammatory cytokines, receptors and transcription factors were differentially expressed (**Table S3**). For example, activating receptors *NCR1* and *KLRK1*, cytolytic granule *CTSC* and transcription factor *TBX21* were not dysregulated; gene expression was confirmed by RT-qPCR (**Fig. S2**). These results suggest a precise and selective regulation of NK cell molecules and inflammatory properties of the innate and acquired immune system during acute stress, which are not accounted solely by an influx of NK cells into the periphery.

3.4 Identification of molecular alterations beyond NK cell subset differences post-landing

To account for NK cell type differences underlying differential gene expression changes from baseline to post-landing, a linear regression model was created taking into account expression of major NK cell markers. In total, four NK cell markers were selected that were consistently found across three different cell type-specific expression databases (Abbas et al., 2005; Shoemaker et al., 2010; Watkins et al., 2009): *CLIC3*, *KLRF1*, *KIR2DL3* and *KIR3DL1*. Accounting for NK cell type composition at post-landing revealed ~15% of the previously identified differentially expressed genes remained significant. Genes encoding for *FOS* and *GZMB* were among the most up-regulated genes surviving this correction, whereas *CLC* and *PAPSS1* were among the most down-regulated (**Table S2**). Functional enrichment analysis revealed that genes corresponding to NK cell mediated cytotoxicity and graft-vs.-host pathways were no longer significant. However, a significant up-regulation of genes enriched for *IL-12* mediated signaling (*FOS, RELB, CD247, GZMB, IL2RB*), cytotoxic T-lymphocyte (CTL) mediated immune response (*CD247, PRF1, GZMB*) and downstream signaling in naive $CD8^+$ T cells remained significant albeit to a lesser extent **(Table S2E)**. A most

interesting finding resulting from this correction was a significant enrichment of genes specific to the adrenal cortex, a key mediator of the stress response **(Fig. S3)**.

3.5 Identification and functional annotation of gene co-expression modules

To identify coordinately expressed genes (modules) involved in the short-term variable immune response to acute stress, unsupervised WGCNA was performed. The analysis identified 19 distinct co-expression modules and 1 module representing all background genes that could not be clustered into any module **(Fig. S4),** each with a distinct expression pattern across all four time-points. Subsequently, all modules were functionally annotated using the top significant biological process, pathway and cell type for each individual module (all Bonferroni $p < 0.05$) **(Table S4).**

3.6 Functional gene co-expression modules correlate with stress induced changes in stress hormones

Next, we sought to determine the relationships between the 20 modules identified above and the observed physiological and hormonal fluctuations throughout the stress response. To integrate these multi-scale data types, module eigengene (ME) values were correlated to each time-point and all recorded subjective and physiological traits (**Fig. S5**). Briefly, ME value is the first PC of module expression and summarizes the main trend of expression within a module. Among the modules with high association with time-points and physiological traits, the *ME* of a module specific for 'Cytokine Production' was negatively correlated to post-landing (r = -0.29, p = 0.05) as well as fluctuations in circulating norepinephrine (r = -0.32, p = 0.03). The ME of modules associated to 'T Cell Receptor (TCR) Signaling Pathway' and 'NK Cell Mediated Cytotoxicity' were positively correlated to post-landing (r = 0.28, p = 0.06; r = 0.57, p = 4E-05 respectively). Moreover, the 'NK Cell Mediated Cytotoxicity' module was positively correlated to norepinephrine (r = 0.39, p = 0.007) which was expected given elevated norepinephrine and NK cell specific gene expression peak post-landing and

return to baseline levels one-hour later (**Fig. 1D** & **Fig. 2C**). Of interest, the expression pattern of each marker gene used in our linear model to correct differential gene expression analysis (*CLIC3*, *KLRF1*, *KIR2DL3* and *KIR3DL1*) showed strong correlation to the ME of this particular module, confirming that the genes for our linear model were appropriately chosen. The ME of a 'Hemostasis' module showed gradual change from negative to positive correlation from baseline to one-hour post-landing and was significantly correlated to beta-endorphin fluctuations (r = 0.32 p = 0.03). Additionally, the ME for a module involved in 'Oxygen Uptake and Carbon Dioxide Release' was positively correlated to heart rate (r=0.38, p=0.01) and salivary cortisol levels (r=0.43, p=0.003), highlighting the interaction between the cardiovascular and respiratory systems. Most interestingly, including gender as a discrete measure revealed that many modules were either positively or negatively correlated to gender differences (**Fig. S5**) suggesting gender-specific expression patterns within each of these modules.

3.7 Gender-specific peripheral immune activation evident by divergent expression profiles within functional co-expression modules

The extent of co-expression differences was visualized throughout the stress response considering gender, averaging ME values for seven males and six females at each time-point. A Bayes ANOVA was used to compare *ME* expression values for modules of interest across time-points while taking into account gender differences (**Fig. 3**). The 'NK cell mediated cytotoxicity' and 'Ribosome Biogenesis' modules showed intensified expression post-landing in males relative to females (**Fig. 3A-B**), whereas the expression of the 'TCR Signaling Pathway' module was highest one-hour post-landing in males relative to females (**Fig. 3C**). Co-regulated genes specific to 'Hemostasis', which includes genes for blood coagulation, showed a gradual increase in expression (**Fig. 3D**) for both males and females peaking one-hour post-landing relative to baseline. Strikingly, four modules specific to 'Immune/Defense Response', 'Response to Wounding', 'Cytokine Production' and 'Interferon Signaling' (**Fig. 3E-H**) were down-

regulated in males post-landing and one-hour post-landing relative to females, while ME expression either increased or remained unchanged.

3.8 Stress induces changes in leukocyte and lymphocyte subset differential counts

Acute stress has been shown to cause a redistribution of leukocytes throughout the periphery (Dhabhar, 2009). To fully characterize changes in peripheral leukocyte and lymphocyte subsets throughout acute psychological stress in the present study, a second cohort consisting of 26 participants (17 male and 9 female) was recruited under the same matching experimental design as the gene expression cohort. Subsequent blood samples were subjected to flow cytometry analysis. These quantitative cell-type data were also used to better understand the extent of which gene expression results may be affected by migrating cell types. Changes within leukocyte and lymphocyte subsets were measured and displayed as both percentages and absolute cell counts combined across both males and females **(Fig. 4)**, as there were no strong differences in cell type fluctuations between genders **(Table S5)**.

Total leukocytes significantly increased from baseline to pre-boarding and post-landing, returning to baseline levels one-hour post-landing. There was a marked increase in the proportion and absolute count of neutrophils pre-boarding, while the post-landing proportion, albeit significantly greater than baseline, was significantly smaller than pre-boarding. Eosinophil proportion and absolute count reduced pre-boarding and remained low post-landing and one-hour post-landing relative to baseline. Monocytes and total lymphocytes showed similar patterns with the lowest proportion and absolute cell counts pre-boarding.

Changes in lymphocyte subsets were also investigated (**Fig. 4 & Table S5**). The percentage of $CD19^{+}$ B lymphocytes and absolute B cell numbers were significantly reduced post-landing. Conversely, NK cells (defined as $CD3^{-}CD16^{+}CD56^{+}$) were

significantly increased pre-boarding and post-landing. The percentage of $CD3^+$ T lymphocytes were significantly reduced post-landing while absolute number of T lymphocytes was significantly decreased pre-boarding compared to baseline. Of the $CD3^+$ lymphocytes, $CD8^+$ and $CD4^+$ T cell absolute counts significantly increased post-landing relative pre-boarding, while $CD4^+$ T cell proportions decreased post-landing.

4. Discussion

This study describes the molecular and cellular response of the human innate and acquired immune system in reaction to physical danger. A first-time tandem skydive was used as a short-term longitudinal design to simulate acute psychological stress in a controlled environment; the stressor induces a severe form of emotional response aligned with distress related to fear (Carter and Goldstein, 2011). Our exploratory study took a dual approach. First, comparative analysis of PBL gene expression profiles between time-points identified that most gene expression changes occurred during/immediately after the stress response. Here, immediate immunomodulation is observed as a selective up-regulation of NK cytotoxicity genes, further validated with RT-qPCR assays. Correcting for changes in NK cells post-stressor revealed a molecular signature specific to the adrenal cortex. Second, focusing our analysis on co-expressed modules revealed gender-specific peripheral immune activation evident by hundreds of co-regulated genes within several biologically annotated modules whose expression differed between males and females. These discoveries provide a useful characterization of acute stress-induced immune system alterations with implications for the understanding and treatment of stress-related disorders and gender vulnerability to stress-induced pathologies.

4.1 NK cell stress susceptibility and selective regulation of NK cell cytotoxic signaling

Although our flow cytometry data showed significant changes in leukocyte subtypes in the course of the stressor, we also showed that changes in observed gene expression

profiles cannot be explained solely by the fluctuation of different leukocyte subsets. For example, peripheral neutrophils were elevated and peripheral eosinophils were reduced in the periphery pre-boarding in anticipation of the stressor. The changes in cell composition were paralleled by the up-regulation of 48 genes and the down-regulation of 46 genes, which were not associated to any functional annotations or leukocyte cell type specificity.

One unexpected finding of our study is the selective up-regulation of only a subset of NK cell genes post-landing (confirmed by RT-qPCR **Fig. S2**), despite a pronounced 2.5 fold increase of NK cells in the periphery (**Fig. 4**). The possible implications of this result may be explained through four phenomena. First, it is possible that a subset of NK genes that displayed no change in expression, were down-regulated in individual NK cells. NK cell activity may be regulated post-transcriptionally, including increases in translation and redistribution of receptors to the cell surface, which is a likely mechanism due to a fast nature of the response. Second, it is also possible that a specialized, characterized (e.g. $CD56^{Lo}$ (Bosch et al., 2005)) or not-yet characterized subset of NK cells, expressing only a subset of specific markers is mobilized into the periphery in response to stress. Third, since gene expression was profiled from the mixture of cells, contribution of other leukocyte subsets that express overlapping sets of genes cannot be ruled out. In particular, gene expression markers for $CD8^+$ T cells were slightly elevated post-landing compared to baseline despite no change in $CD8^+$ T cell frequency in blood (**Fig 4**). Even though NK cell-related genes are also expressed at lower levels in these cells, a large change in their expression in T cells can contribute to their expression change in total leukocytes. Finally, although differential gene expression analyses were gender corrected, it could be that that the NK cell response is modulated to differing degrees between males and females as suggested by WGCNA observed gender-specific differences (**Fig 3A**).

While only ~15% of the originally identified differentially expressed genes were found to be dysregulated after correcting for NK cells, the consistent up-regulation of cell toxicity transcript *GZMB* and transcription factor *FOS* was evident. Proteolytic granzymes, such

as *GZMB*, and granulysin delivered from cytotoxic cells via granule exocytosis cause activation of caspase-dependent apoptosis in stressed or pathogenic target cells (Bernard et al., 1999), which helps to explain functional annotations such as CTL mediated immune response and apoptosis following the correction. The up-regulation of *FOS*, an early immediate gene which is turned on in brain (Bernard et al., 1999), blood (Torres and Lotfi, 2007) and adrenal cortex and mediates physiological adrenocorticotropic hormone-induced responses in adrenal cortical cells (Rui et al., 2014; Verstrepen et al., 2008), is consistent with the enrichment of differentially expressed genes following NK cell correction associated with the adrenal cortex and the production of cortisol (**Fig. S3**). This is an important observation and one that may have been difficult to detect if gene expression was measured for each cell type isolated independently.

4.2 Potential roles of IL-12 signaling and TLRs in response to acute stress

The most pronounced effect following multivariate linear regression to adjust for an influx of NK cells into the periphery post-landing, was the consistent up-regulation of genes involved in IL-12 mediated signaling (*CD247*, *FOS*, *GZMB*, *IL2RB*), and the minor production of IFN- . The IL-12 signaling pathway determines the type and duration of innate and adaptive immune response in part by promoting NK cell cytoxicity as well as the differentiation of naive CD4+ T cells into T helper 1 (Th1) cells via the production of IFN- . Here, up-regulation of IL-12 signaling may indicate priming of the pro-inflammatory arm of the immune system. Such immunomodulation creates an advantage during events such as vaccination since a primed pro-inflammatory state is important for vaccine-mediated T cell immune responses, which are induced by most anti-bacterial and anti-viral vaccination strategies (Dhabhar, 2009). Thus, these data suggest a more focused adaptive immune response which under further emotional distress or antigen presentation may provide a cytokine environment favorable for Th1 polarization of the immune system.

These data also show the down-regulation of MyD88-dependent pathway including signaling molecules *MAPK3*, *CHUK* (i.e. *IKK-*) and toll-like receptors (TLRs) 2, 6 and 10. In homeostatic conditions, TLRs lead to NFκB activation and production of pro-inflammatory cytokines IL1 , IL6 and TNF , all involved in different pathways for innate immune activation and defense (Rui et al., 2014; Verstrepen et al., 2008). Down-regulation of TLRs is consistent with previous reports suggesting that increased cortisol levels during acute stress may inhibit the NFκB, JAK-STAT and MAPK signaling pathways (Kadmiel and Cidlowski, 2013; Reichardt et al., 2002; Rui et al., 2014; Webster et al., 2002). Under repeated bouts of acute stress or chronic exposure to psychosocial stress (and continued emotional activation), the response of HPA axis to sustained stress is diminished and subsequently the effectiveness of glucocorticoids (e.g. cortisol) to regulate the inflammatory response is altered as immune cells become insensitive to its regulatory effects (Cohen et al., 2012). Consequently, inflammatory pathways may become activated and initiate a negative feedback loop driving inflammation and promoting the development of many diseases.

4.3 Gender-specificity of the acute stress response at the transcriptional level and implications for stress-induced pathologies more frequent in women

Another unexpected result stemming from our exploratory gene co-expression approach was the gender-specific immune response to acute stress (**Fig. 3**) despite similar cellular and hormonal alterations (**Table S1** & **Table S5**), which may have relevant translational avenues. For example, it is widely accepted that among individuals experiencing chronic mental stress, cardiovascular disease (CVD) affects women more than men and gender-specific effects of mental stress on the heart is a main component of this disparity (Samad et al., 2014). While gender-specific differences in the psychobiological stress response have not been clearly identified, they may provide valuable insight towards understanding the differential cardiovascular risk in men and women. Processes associated to CVD, such as TCR signaling, defense response, response to wounding, cytokine production and interferon signaling (Mehra et al., 2005)

were differently regulated by acute stress in males and females in our study (**Fig. 3**). These findings may help to explain gender-specific predisposition to CVD and emphasize these genes and pathways as potential tools which may be able to measure an entire facet of CVD risk, the impact of maladaptive molecular response to psychological stress in both sexes and among women in particular. Moreover, since many inflammatory disorders that are most common in women, such as autoimmunity, are also exacerbated by psychological stress (Whitacre, 1999), gender differences in cytokine response to stress (**Fig. 3G**) could mark an important underlying mechanism.

It is widely accepted that women, more frequently than men, suffer from chronic forms of stress such as post-traumatic stress disorder (PTSD) (Becker et al., 2007), more frequently than men, yet the reasons for this disparity are not entirely clear. It has been proposed that these differences are not explained solely on the basis of exposure type and/or severity (Sherin and Nemeroff, 2011) and that modulation of sex steroids such as estrogen and progestoreone have implicated changes in neurotransmitter systems involved in the stress response. However, factors other than exposure must play a role in the development of the disorder that might determine gender vulnerability to PTSD, and these may include transcriptomic level differences. While personalized medicine for such ubiquitous pathologies confronts numerous biomedical and financial challenges, gender-based medicine may provide a more appropriate medical platform, at the least for evaluating gender vulnerability to stress-induced pathologies.

4.5 Putative blood-based biomarkers for discriminating anxiety-based stress from related neuropsychiatric and central nervous system (CNS) disorders

An important task for studies investigating peripheral mechanisms of CNS disorders (multiple sclerosis, stroke and seizure) as well as panic attacks, myocardial ischemia, and related rodent models of such disease (Achiron et al., 2004; Kim et al., 2014; Samad et al., 2014; Yang et al., 2005; Yang et al, 2001), is the ability to disentangle molecular mechanisms more closely associated with the clinical presentation of disease rather than differences which are psychogenic in nature. For example, in our study the

most down-regulated gene post-stressor and one-hour post-stressor was *IMAP2*, and the most down-regulated transcription factor post-stressor was *ME2*, as indicated by interactome analysis (**Fig. S1**). In genome-wide studies, both genes *IMPA2 and ME2* have been reported as susceptibility genes in febrile seizures and idiopathic generalized epilepsy (Arai et al., 2007; Greenberg et al., 2005; Mas et al., 2004; Prasad et al., 2013). Recently, seizures have been reported to occur following acute emotional stress (i.e. psychogenic non-epileptic seizures) rather than the result of abnormal electrical activity in the brain, as with epilepsy (Testa et al., 2012). However, baseline human blood gene expression signatures of epilepsy prior to drug treatment do not include dysregulation of *IMPA2* or *ME2* (Piro et al., 2011; Yang et al., 2005; Yang et al, 2001). Moreover, dysregulation of these genes was not observed in the brains of rodents post-seizure (Harald et al., 2001). While these results should be interpreted cautiously, the general inconsistencies between these studies and the results presented here may provide evidence for a role of *IMPA2* and *ME2* in differentiating between psychogenic non-epileptic seizures from true epileptic seizures.

4.6 Hypoxia does not contribute to observed gene expression profiles

Studies using an exaggerated 12 hours sustained poikilocapnic hypoxic model system have noted the dysregulation of mRNA expression specific to hypoxia-inducible factor 1 (*HIF1A), GAPDH*, *EPO*, and *VEG* within the first two-hours (Pialoux et al., 2009). Thus, there was a slight possibility that factors attributable to a short-term exposure (i.e. 20 minutes) to high altitude (i.e. 13,000 ft.), such as hypobaric hypoxia, could influence gene expression in subjects during the skydive. Therefore, the expression of these mRNA species was investigated. *HIF1A* was measured on the microarrays by three probes: none of these probes were detected as significant in our differential gene expression analysis (all $p > 0.1$). None of the probes for other genes associated with hypoxic conditions such as *GAPDH*, *EPO* or *VEG* (Pialoux et al., 2009; Zhong et al., 1999) were dysregulated. We did observe the differential expression of *HIPK2* among the identified anticipatory genes at pre-boarding, known to suppress *HIF1A* in hypoxia-mimicking conditions (Nardinocchi et al., 2009). The early activation of *HIPK2* may

reflect increased anticipatory heart rate and early rapid breathing in anticipation to the skydive which may be working to suppress 'hypoxia-mimicking' conditions in the PBL microenvironment.

4.7 Limitations and future direction

While we adjusted for cell type changes affecting global gene expression, clear limitations of this study are the lack of transcriptomic investigation on individual cell types and the ability to perform transcriptomic analysis and flow cytometric data analysis on the same cohort of individuals. While gender specific differences were observed across a small number of samples, the evidence of hundreds of co-expressed functional modules throughout the skydive is significantly robust. However, one important future direction would be to extend and replicate this exploratory study using a larger cohort of participants.

4.8 Conclusion

Molecular mechanisms underlying the rapid adaptation of the immune system to an acute stressor are still incompletely defined. Our exploratory study profiled the PBL transcriptome throughout a first-time tandem skydive, as a measure of intense acute psychological stress, to reveal a detailed response to acute stress at the molecular level. A novel finding of the study is the degree of specificity of the immune response with respect to upregulation of a subset of NK cell genes that cannot be solely attributed to the influx of NK cells into the periphery in response to stress parallel by increases in cortisol and catecholamines. Correcting differential gene expression analysis post-stressor revealed a molecular signature specific to the adrenal cortex. Network analysis stratified by gender identified hundreds of genes within several functional co-expression modules responding to stress in a gender-specific manner. These results offer a spring-board for future research aimed towards identifying therapeutic targets of stress-related disorders, while underscoring the importance of gender-specific molecular profiles

which could be used to better understand gender vulnerability to stress-induced disease.

Data Availability

The microarray data have been submitted to the NCBI Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo/) under accession number GSE69172.

Authors' contribution

BKR, LMP and WYE conceived and designed the study. LMP and JMC recruited the participants and conducted phenotyping and samples collection on the skydivers. WYE conducted flow cytometry assays. CHW provided gene expression guidance. NBB prepared RNA samples and conducted gene expression assays. MSB and NBB conducted gene expression data analysis and interpretation of data. All authors contributed to writing the manuscript.

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References

1. Abbas AR, Baldwin D, Ma Y, Ouyang W, Gurney A, Martin F et al. 2005. **Immune Response in Silico (IRIS): Immune-specific Genes Identified from a Compendium of Microarray Expression Data.** *Genes and Immunity* 6.4:319-31.
2. Achiron A, Gurevich M, Friedman N, Kaminski N, Mandel M. 2004. **Blood Transcriptional Signatures of Multiple Sclerosis: Unique Gene Expression of Disease Activity**. *Annals of Neurology* 55.3: 410-17.
3. Ackerman KD, et al. 2002. **Stressful life events precede exacerbations of multiple sclerosis**. *Psychosom Med* 64:916-20.
4. Al'Abadie MS, Kent GG, Gawkrodger DJ. 1994. **The relationship between stress and the onset and exacerbation of psoriasis and other skin conditions**. Br *J Dermatol* 130:199-203.
5. Altemus M, Rao B, Dhabhar FS, Ding W, Granstein RD. 2001. **Stress-induced changes in skin barrier function in healthy women**. *J. Invest. Dermatol*. 117:309-317.
6. Amkraut AA, Solomon CF, Kraemer HC. 1971. **Stress, early experience and adjuvant-induced arthritis in the rat**. *Psychosom* Med 33: 203-14.
7. Arai R, Ito K, Ohnishi T, Ohba H, Akasaka R, Bessho Y et al. 2007. **Crystal Structure of Human Myo-inositol Monophosphatase 2, the Product of the Putative Susceptibility Gene for Bipolar Disorder, Schizophrenia, and Febrile Seizures**. *Proteins: Structure, Function, and Bioinformatics* 67(3):732-42.
8. Becker JB, Monteggia LM, Perrot-Sinal TS, Romeo RD, Talylor JR et al. 2007. **Stress and disease: is being female a predisposing factor?** *J Neurosci*. 27:11851-11855.
9. Bernard K, Cambiaggi A, Guia S, Bertucci F, Granjeaud S, et al. 1999. **Engagement of Natural Cytotoxicity Programs Regulates AP-1 Expression in the NKL Human NK Cell Line**. *The Journal of Immunology* 162(7):4062-4068.
10. Black PH. 2006. **The inflammatory consequences of psychologic stress: Relationship to insulin resistance, obesity, atherosclerosis and diabetes mellitus, type II**. *Medical Hypotheses* 67(4):879-891.
11. Bosch JA, Berntson GG, Cacioppo JT, Dhabhar FS, Marucha PT. 2003. **Acute stress evokes selective mobilization of T cells that differ in chemokine receptor expression: a potential pathway linking immunologic reactivity to cardiovascular disease**. *Brain Behav Immun* 17(4):251-259.

12. Bosch JA, Berntson GG, Cacioppo JT, Marucha PT. 2005. **Differential Mobilization of Functionally Distinct Natural Killer Subsets During Acute Psychological Stress**. *Psychosomatic Medicine* 67(3):366-375.
13. Carter JR, Goldstein DS. 2011. **Sympathoneural and Adrenomedullary Responses to Mental Stress**. *Comprehensive Physiology* 119-46.
14. Charney DS. 2004. **Psychobiological mechanisms of resilience and vulnerability: implications for successful adaptation to extreme stress**. *Am J Psychiatry* 161:195-216.
15. Chen J, Bardes EE, Aronow BJ, Jegga AG. 2009. **ToppGene Suite for gene list enrichment analysis and candidate gene prioritization.** *Nucleic Acids Res*. 37:W305-11.
16. Cohen S, Janicki-Deverts D, Doyle W, Miller GE, Frank E et al. 2012. **Chronic Stress, Glucocorticoid Receptor Resistance, Inflammation, and Disease Risk**. *Proceedings of the National Academy of Sciences* 109(16):5995-999.
17. Dhabhar FS, Miller AH, McEwen BS, Spencer RL. 1995. **Effects of stress on immune cell distribution-dynamics and hormonal mechanisms**. *J Immunol* 154:5511-27.
18. Dhabhar FS. 2009. **Enhancing versus Suppressive Effects of Stress on Immune Function: Implications for Immunoprotection and Immunopathology**. *Neuroimmunomodulation* 16.5:300-17.
19. Du P, Kibbe WA, Lin SM. 2008. **Lumi: a pipeline for processing Illumina microarray**. *Bioinformatics* 24(13):1547-1548.
20. Garg A, Chren MM, Sands LP, Matsui MS, Marenus KD, Feingold KR et al. 2001. **Psychological stress perturbs epidermal permeability barrier homeostasis: implications for the pathogenesis of stress- associated skin disorders**. *Arch Dermatol* 137:53-9.
21. Greenberg DA, Cayanis E, Strug L, Marathe S, Durner M, Pal DK et al. 2005. **Malic enzyme 2 may underlie susceptibility to adolescent-onset idiopathic generalized epilepsy**. *Am. J. Hum. Genet.* 76:139-46.
22. Harald S, Badan I, Fischer B, Wagner AP. 2001. **Dynamics of Gene Expression for Immediate Early- and Late Genes after Seizure Activity in Aged Rats**. *Archives of Gerontology and Geriatrics* 32(3):199-218.
23. Kadmiel M, Cidlowski JA. 2013. **Glucocorticoid receptor signaling in health and disease**. *Trends in Pharmacological Sciences* 34(9):518-530.
29. Kayala MA, Baldi P. 2012. **Cyber-T Web Server: Differential Analysis of High-throughput Data**. *Nucleic Acids Research* 40.W1:W553-559.
30. Kim J, Ghasemzadeh N, Eapen DJ, Chung NC, Storey JD et al. 2014. **Gene expression profiles associated with acute myocardial infarction and risk of cardiovascular death**. *Genome Med*. 6:40.
31. Langfelder P, Horvath S. 2008. **WGCNA: an R package for weighted correlation network analysis**. *BMC Bioinformatics* 9:559.
32. Mas C, Taske N, Deutsch S, Guipponi M, Thomas P, Covanis A, et al. 2004. **Association of the connexin36 gene with juvenile myoclonic epilepsy**. *J. Med. Genet*. 41:e93.
33. Mathers C, Fat DM, Boerma J. 2008. **The Global Burden of Disease: 2004 Update**. Geneva, Switzerland: World Health Organization.

34. Mehra VC, Ramgolam VS, Bender JR. 2005. **Cytokines and Cardiovascular Disease**. *Journal of Leukocyte Biology* 78(4):805-18.
35. Miller J, Woltjer RL, Goodenbour JF, Horvath S, Geschwind DH. 2013. **Genes and pathways underlying regional and cell type changes in Alzheimer's disease**. *Genome Medicine* 5:48.
36. Nardinocchi L, Puca R, Sacchi A, D'orazi G. 2009. **Inhibition of HIF-1alpha Activity by Homeodomain-interacting Protein Kinase-2 Correlates with Sensitization of Chemoresistant Cells to Undergo Apoptosis**. *Molecular Cancer* 8(1).
38. Pialoux V, Mounier R, Brown AD, Steinback CD, Rawling JM et al. 2009. **Relationship between oxidative stress and HIF-1_ mRNA during sustained hypoxia in humans**. *Free Radical Biology and Medicine* 46(2):321-326.
39. Piro RM, Molineris I, Ala U, Di Cunto F. 2011. **Evaluation of Candidate Genes from Orphan FEB and GEFS Loci by Analysis of Human Brain Gene Expression Atlases**. Ed. Takeo Yoshikawa. PLoS ONE 6(8):e23149.
40. Prasad DK, Satyanarayana U, Munshi A. 2013. **Genetics of Idiopathic Generalized Epilepsy: An Overview**. *Neurology India* 61(6): 572.
41. Reichardt HM, Tuckermann JP, Göttlicher M et al. 2002. **Repression of inflammatory responses in the absence of DNA binding by the glucocorticoid receptor**. *EMBO Journal* 20(24):7168-7173.
43. Rui Tian T,Hou G,Li D, Yuan TF. 2014. **A Possible Change Process of Inflammatory Cytokines in the Prolonged Chronic Stress and Its Ultimate Implications for Health**. *The Scientific World Journal Article* 1155:780616.
44. Samad Z, Boyle S, Ersboll M, Vora AN, Zhang Y et al. 2014. **Sex Differences in Platelet Reactivity and Cardiovascular and Psychological Response to Mental Stress in Patients With Stable Ischemic Heart Disease: Insights From the REMIT Study**. *J Am Coll Cardiol.* 64(16):1669-1678.
45. Schedlowski M, Jacobs R, Stratmann G, Richter S, Hadicke A et al. 1993. **Changes of natural killer cells during acute psychological stress**. *J Clin Immunol* 13(2):119-126.
46. Segerstrom SC, Miller GE. 2004. **Psychological stress and the human immune system: a meta-analytic study of 30 years of inquiry**. *Psychol. Bull* 130: 1-37.
47. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT et al. 2003. **Cytoscape: a software environment for integrated modules of biomolecular interaction networks.** *Genome Research* 13(11):2498-504.
48. Sherin JE, Nemeroff CB. 2011. **Post-traumatic stress disorder: the neurobiological impact of psychological trauma.** *Dialogues in Clinical Neuroscience* 13(3):263-278.
49. Shoemaker JE, Fukuyama S, Sakabe S, Kitano H, Kawaoka Y. 2011. **CTen: a web-based platform for identifying enriched cell types from heterogenous microarray data**. *BMC Genomics* 13:460.
50. Smyth GK. 2005. **Limma: linear models for microarray data**. In Gentleman R, Carey V, Dudoit S, Irizarry R and Huber W Eds., Bioinformatics and Computational Biology Solutions Using R and Bioconductor (Springer, New York), pp. 397-420.

52. Testa SM, Krauss GL, Lesser RP, Brandt J. 2012. **Stressful Life Event Appraisal and Coping in Patients with Psychogenic Seizures and Those with Epilepsy**. *Seizure* 21(4):282-87.
53. Torres TEP and Lotfi CFP. 2007. **Distribution of cells expressing Jun and Fos proteins and synthesizing DNA in the adrenal cortex of hypophysectomized rats: regulation by ACTH and FGF2**. *Cell and Tissue Research* 329(3): 443-455.
54. van Westerloo DJ, Choi G, Lowenberg EC, Truijen J, de Vos AF, et al. 2001. **Acute stress elicited by bungee jumping suppresses human innate immunity**. *Mol. Med.* 17, 180-188
55. Verstrepen L, Bekaert T, Chau LT, Tavernier J, Chariot A, Beyaert R. 2008. **TLR-4, IL-1R and TNF-R signaling to NF-_B: variations on a common theme**. *Cellular and Molecular Life Sciences* 65(19): 2264-2978.
59. Watkins NA, Gusnanto A, De Bono B, De S, Miranda-Saavedra et al. 2009. **A HaemAtlas: Characterizing Gene Expression in Differentiated Human Blood Cells**. *Blood* 113.19: E1-E9.
60. Webster JI, Tonelli L, and Sternberg EM. 2002. **Neuroendocrine regulation of immunity**. *Annual Review of Immunology* 20: 125-163.
61. Whitacre CC. 1999. **A gender gap in autoimmunity**. *Science* 283:1277-1278.
63. Yang T, Gilbert DL, Glauser TA, Hershey AD, Sharp FR. 2005. **Blood Gene Expression Profiling of Neurologic Diseases**. *Archives of Neurology Arch Neurol* 62(2): 210.
64. Yang T, Lu A, Aronow BJ, Sharp FR. 2001. **Blood Genomic Responses Differ after Stroke, Seizures, Hypoglycemia, and Hypoxia: Blood Genomic Fingerprints of Disease**. *Annals of Neurology Ann Neurol*. 50(6):699-707.
65. Zhong H, Simons JW . 1999. **Direct Comparison of GAPDH, Beta-Actin, Cyclophilin, and 28S rRNA as Internal Standards for Quantifying RNA Levels under Hypoxia**. *Biochemical and Biophysical Research Communications* 259(3):523-26.

Main Figure Legends

Figure 1. Physiological changes observed throughout the sequence of events leading up to, during, and after a first time tandem skydive jump. (**A**) The skydiving paradigm and relevant time-points. (**B**). Heart rate measurements (bpm) were obtained throughout the course of both baseline and skydive days. (**C**) Salivary cortisol (pg/ml) was collected every 15 minutes from 9:15 am until 11:45 am on both baseline and skydive days. (**D**) Norepinephrine (pg/ml) and (**E**) epinephrine (pg/ml) were measured in duplicate and averaged at the corresponding four time points. Dark blue represents baseline day and light-blue represents skydive day. Error bars represent 95% confidence interval and (*) indicates p-value < 0.05 based on non-parametric Mann-Whitney U test. All p-values are reported in **Table S1**.

Figure 2. Comprehensive depiction of gender corrected differentially expressed genes (all $p < 0.01$) leading up to and following acute psychological stress. (**A**) Volcano plots for differentially expressed genes display extent of log fold-change compared to the –log10 p-value significance at pre-boarding, post-landing and one-hour post-landing respective to baseline. (**B**) Overlap of down-regulated and up-regulated genes across time-points. (**C**) All differentially expressed genes identified from baseline to post-landing. (**D**) Functional annotation of differentially expressed genes identified baseline to post-landing performed separately for up- and down-regulated genes. The top 4 most significant annotations (all $p < 0.05$ Bonferroni corrected) are shown for categories of biological processes and pathways (annotated with ToppGene) and cell types (annotated with CTen). Genes involved in IL-12 signaling and MyD88-dependent pathway are displayed for quick referencing.

Figure 3. Gender specific differences in functional gene co-expression modules. ME values for modules of interest are evaluated across the four time-points comparing males and females. Modules specific to (**A**) NK cell cytoxicity, (**B**) ribosome biogenesis, (**C**) TCR signaling pathway, (**D**) hemostasis, (**E**) immune/defense response, (**F**) response to wounding, (**G**) cytokine production (**H**), interferon/cytokine signaling are displayed. Heatmaps display the extent to which expression profiles of the top 10 functional hub genes, for each corresponding module, change in males and females across different time-points. White line spacers in heatmaps indicate the four time-points. The functional annotation and number of genes within each module are displayed above the boxplots. A Bayes ANOVA was used on ME values to test for significance between males and females, (**) indicates $p < 0.001$ implying strong gender-specific differences throughout course of the stress response.

Figure 4. A quantitative measurement of the PBL cell lineage via flow cytometry. The analysis used a gating strategy based on the forward scatter/side characteristics of immune cells from total leukocytes; granulocytes ($CD45^+$), monocytes ($CD14^+$), T cells ($CD3^+$, $CD4^+$, $CD8^+$), B lymphocytes ($CD19^+$) and NK cells ($CD3^-CD56^+CD16^+$). The raw

flow data is presented as a percentage of gated cells (as indicated by the bar plots). To determine the absolute immune cell counts (as indicated by the line), leukocyte differential counts from the complete blood counts results were used to produce estimates of the actual number of immune cells in the peripheral blood samples. Statistical analysis was based on a Dunnet's Test multiple comparison of means was used, comparing measurements back to baseline. All corresponding p-values are presented in **Table S5**.

Supplementary Figure Legends

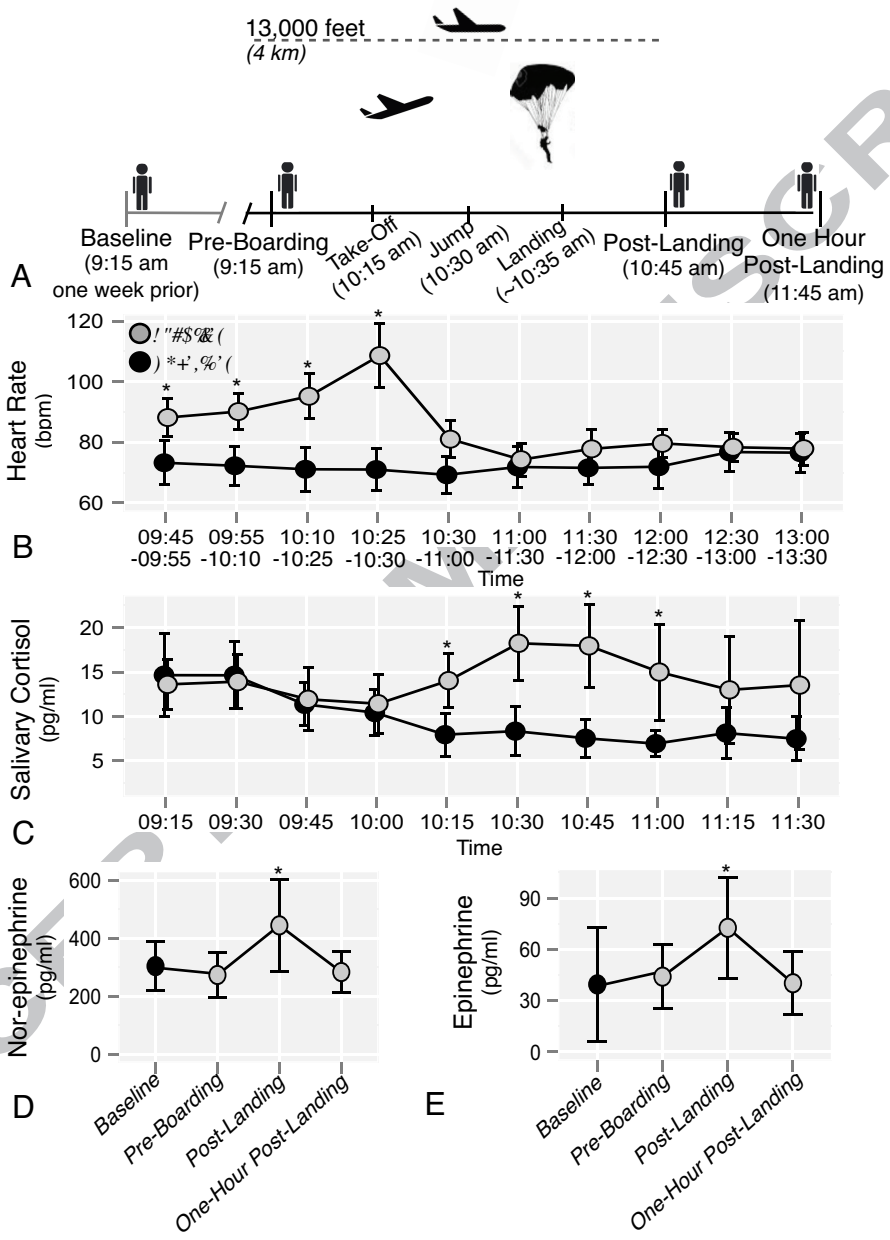
Figure S1. Protein interaction network (PIN) of differentially expressed genes. This PIN reflects differentially expressed genes pre-boarding, post-landing, and one-hour post-landing as delineated by the pie chart. Large node sizes reflect key transcription factors with more than 10 validated interactions. Red, up-regulation; blue, down-regulation, on the scale presented by the color bar; white, no change. Purple circle, genes related to innate immunity and green circle, genes related to acquired immunity.

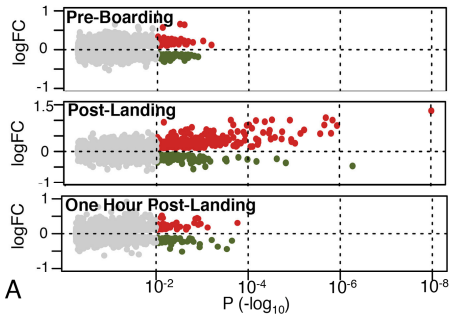
Figure S2. RT-qPCR confirmation of differentially expressed genes. Gene expression analysis by RT-qPCR was performed for 18 genes found as differentially expressed between baseline and post-landing, three genes that were not differentially expressed and one gene that was below the limit of detection in the microarray analysis. •Ct values ($Ct - Ct_{GAPDH}$) were calculated for each gene at baseline (white bars) and post-landing (black bars). Error bars represent standard deviation. The smaller •Ct values indicate presence of greater number of mRNA molecules in a sample. All the differentially expressed genes identified by microarray were also differentially expressed by RT-qPCR (** $p < 0.01$, *$0.01 < p < 0.05$) between baseline and post-landing time points. All genes that were not differentially expressed by microarray, were also not differentially expressed by RT-qPCR (ns, not significant).

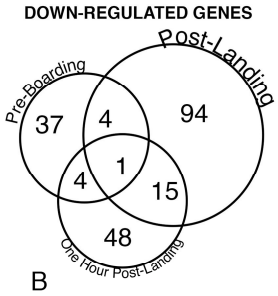
Figure S3. CTen cell type enrichment analyses based on differentially expressed genes identified from baseline to post-landing. (**A**) Using all identified genes from Figure 2C, without correcting for NK cell types. (**B**) Enrichment using genes that survived cell type correction with multivariate linear model.

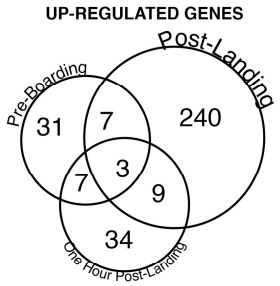
Figure S4. Identification and organization of gene co-expression modules. WGCNA cluster dedrogram and network modules with corresponding information bars. The network was raised to the beta power of 9 to satisfy scale-free topology. The bar represents the identified modules (as denoted by colors), the grey module corresponds to genes which do not cluster into any other module. Each line represents a gene (leaf), and multiple genes clustered together represent a group of co-regulated genes (low hanging branches) on the cluster dendrogram (tree). The *y*-axis corresponds to distance determined by the extent of topological overlap measure (1-TOM).

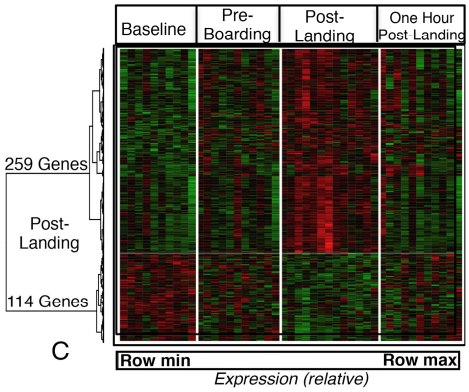
Figure S5. Functional PBL gene module – stress hormone relationships throughout the stress response. ME values of functional PBL gene modules were correlated to underlying stress hormone measurements. The measure of correlation, *r*, is the top value in each box and the related *p*-value is designated below within brackets. Red signifies a positive correlation and blue signifies a negative correlation as indicated by scale. The number next to each functional PBL gene module signifies the number of genes within that module with kME > 0.05 used for functional annotation. Abbreviations: NGF, nerve growth factor; NK, natural killer.











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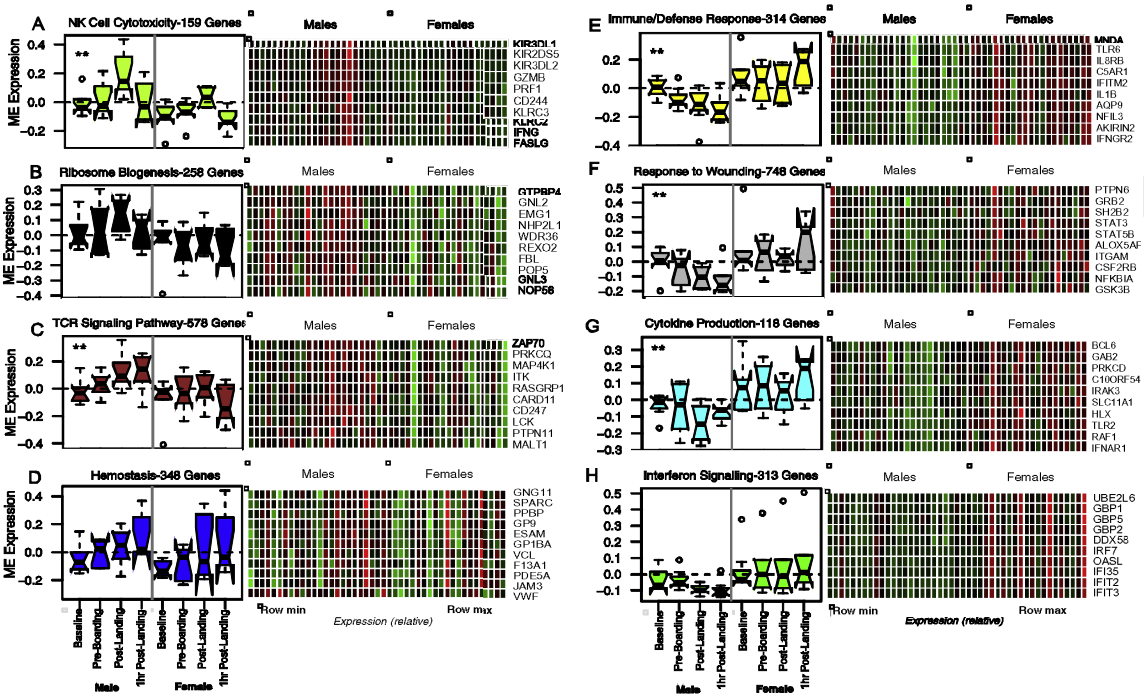
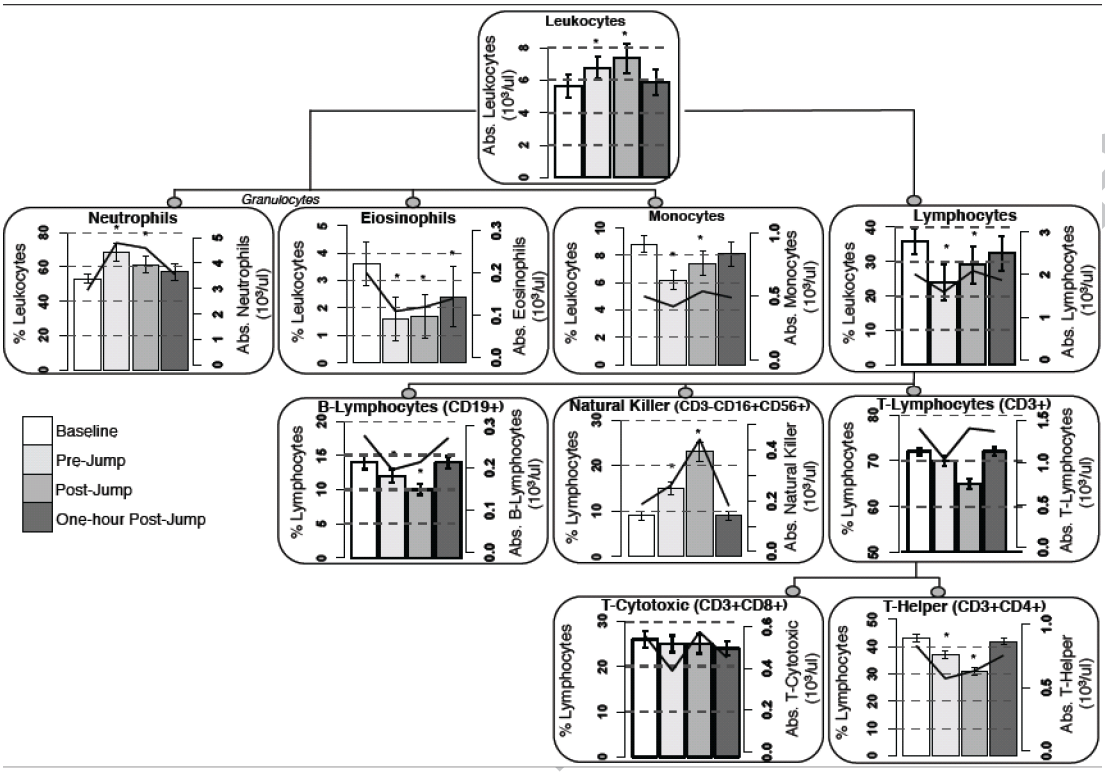
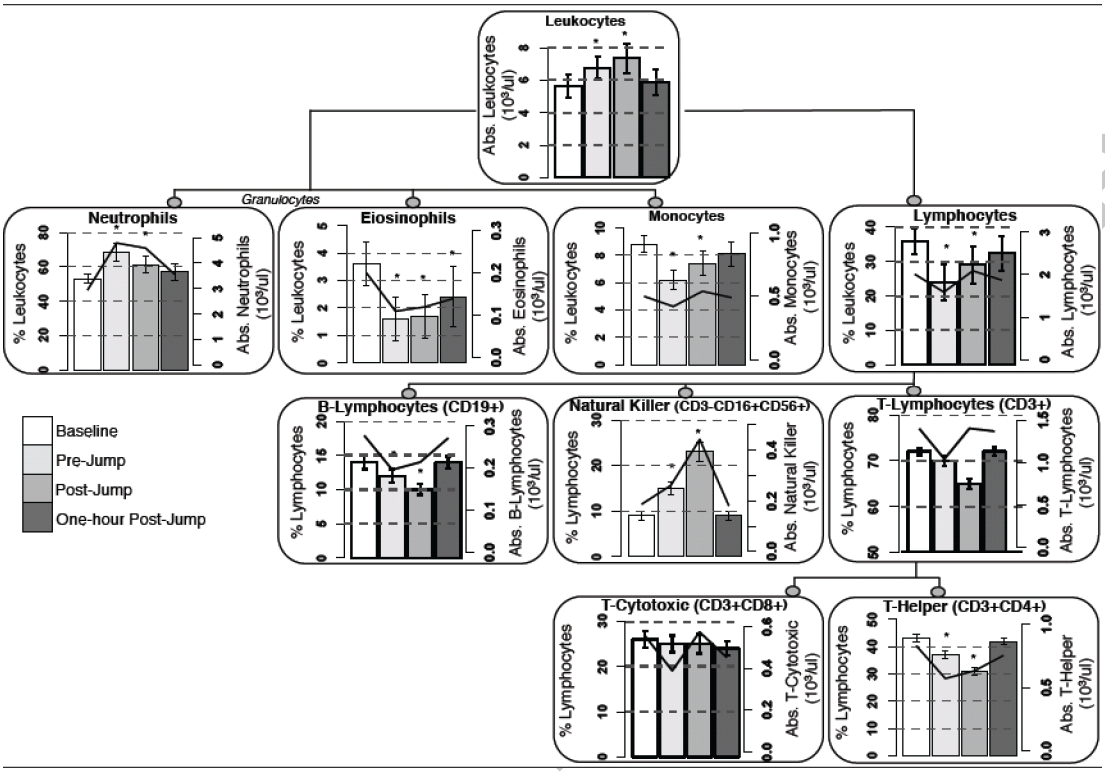
		Functional Annotations	Bonferroni *P*	Genes(*n*)
Up-Regulated	Biological Processes	Immune response	6.9E-07	45 / 1416
		Regulation of immune response	7.7E-06	31 / 801
		Regulation of immune system process	1.0E-04	37 / 1212
		Response to lipid	7.0E-04	26 / 719
	Pathways	Natural killer cell mediated cytotoxicity	9.1E-10	17 / 135
		Graft-versus-host disease	4.1E-06	9 / 34
		IL12-mediated signaling events	7.8E-05	9 / 62
		Downstream signaling in naive CD8+ T	2.0E-04	8 / 50
	Cell-Type	CD56+ NK-Cells	2.0E-26	84 / 870
		CD8+ T-Cells	3.0E-06	43 / 617
Down-Regulated	Biological Processes	MyD88-dependent toll-like receptor signaling	5.7E-03	6 / 84
		Leukocyte activation involved in immune response	5.8E-03	7 / 88

CCL4L2
CD247
EOMES
FASLG
FOS
GZMA
GZMB
IL12RB1
IL2RB
BTK
CHUK
MAPK3
TLR2
TLR10
TNIP1







Highlights

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- We modeled acute psychological stress induced by first-time tandem skydive
- Blood transcriptome, physiological and flow-cytometry measurements were collected
- Early immunomodulation was manifested by selective up-regulation of NK-cell genes
- Cell-type correction revealed a molecular signature specific to the adrenal cortex
- Hundreds of co-regulated genes demonstrate gender specific immune response profiles

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