Brain, Behavior, & Immunity - Health 9 (2020) 100145

Contents lists available at ScienceDirect

Brain, Behavior, & Immunity - Health

journal homepage: www.editorialmanager.com/bbih/default.aspx

Full Length Article

Exploratory RNA-seq analysis in healthy subjects reveals vulnerability to viral infections during a 12- month period of isolation and confinement

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ARTICLE INFO

Keywords:
Stress
Isolation
Confinement
RNA-seq
Antiviral immunity

ABSTRACT

Exposure to stressful environments weakens immunity evidenced by a detectable reactivation of dormant viruses. The mechanism behind this observation remains unclear. We performed next generation sequencing from RNA extracted from blood samples of 8 male subjects collected before, during and after a 12-month stay at the Antarctic station Concordia. RNA-seq data analysis was done using QIAGEN Ingenuity Pathway Analysis (IPA) software. Data revealed the inactivation of key immune functions such as chemotaxis and leukocyte recruitment which persisted after return. Next to the activation of the stress response eIF2 pathway, interferon signaling was predicted inactivated due to a downregulation of 14 downstream genes involved in antiviral immunity. Among them, the interferon stimulated genes (ISGs) IFITM2 and 3 as well as IFIT3 exhibited the strongest fold changes and IFIT3 remained downregulated even after return. Impairment of antiviral immunity in winter-over crew can be explained by the downregulation of a battery of ISGs.

1. Introduction

Prolonged periods of isolation and confinement, whether it be in a group of people or complete self-isolation, are stressors that disbalance body and mind. The observed negative effects range from mood and sleep disturbances to cardiovascular and immune dysfunction (Xia and Li, 2018; Yi et al., 2014). Since the early days of human space exploration, isolation and confinement of astronauts, living and working in a very stressful environment for up to twelve months, have been acknowledged as risk factors for mission success and crew health (Walters and Henning, 1961; Schmitt and Schaffar, 1993). Due to the low number of subjects, limited crew time and various technical constraints, several high fidelity ground models, so called space analogs, with unique characteristics exist. These allow isolating single aspects of the space environment on Earth and make space research more accessible (Pagel and Chouker, 2016). One of the analog platforms similar to a space station, is the French-Italian *Concordia* Station remotely located on the Antarctic plateau 3233 m above sea level. Participants of winter-over campaigns face 12-months of isolation and confinement under hypobaric hypoxia (~650 hPa) and extreme outside temperatures that can drop down to −80 °C. Further stressors include nutritional changes and circadian rhythm disruption due to seasonal shifts of complete daylight (December, January) to complete darkness (June, July). While astronauts undergo years of specialized training, winter-over crews receive a medical and psychological screening for eligibility only. Thus, this is an excellent model to study the chronic effects of stress, isolation, and confinement on people unbiased by preexisting training, adaptation, or disease (see also http://www.esa

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https://doi.org/10.1016/j.bbih.2020.100145
Received 4 August 2020; Received in revised form 7 September 2020; Accepted 16 September 2020
Available online 24 September 2020

.int/Science_Exploration/Human_and_Robotic_Exploration/Concordia/The_remotest_base_on_Earth).

Previous work has shown that chronic exposure to this stressful environment resulting in the release of stress hormones can preclude effective immune responses and promote hypersensitivity (Feuerecker et al., 2014, 2019; Strewe et al., 2018). Furthermore, a reactivation of *Herpes virus* (EBV) was found in the saliva of winter-over crew (Mehta et al., 2000), and in astronauts, where EBV, VZV and CMV shedding was quantified (Mehta et al., 2000, 2014). Dating back from Bonneau's report in the late last century (Bonneau et al., 1990) mostly clinical studies over the past two decades have revealed that chronic stressors in general and including prolonged isolation are potent conditions to affect the human immune system and its humoral and cellular functions. As a result e.g. chronic infections, cancer, or autoimmune disease can occur (McEwen, 1998; Reiche et al., 2004; Cohen et al., 2007; Glaser and Kiecolt-Glaser, 2005). The effects of stress can even result in brain structural changes and have been also recently quantified as an effect of Antarctic overwintering (McEwen et al., 2012; Stahn et al., 2019). This link between stress and health and viral reactivation and that exposure to stress can specifically affect immune response to virus was reported by Glaser (Glaser and Kiecolt-Glaser, 2005; Glaser et al., 2005) and that high level of stress, e.g. as in extreme daily work or other environmental conditions, can induce herpes virus shedding and increase the risk of viral infection (Coskun et al., 2010; Prather et al., 2018; Pierson et al., 2005; Rooney et al., 2019). Though this reactivation is linked to a higher individual stress response, the mechanism remains unresolved. To elucidate the susceptibility of crew members for infections on the transcriptomic level, we recruited eight male participants of the 2017 Antarctic winter-over campaign at *Concordia* station and performed transcriptome profiling of RNA-seq data before, after and on four timepoints during isolation. We used the RNA-seq transcriptomic approach because it allows us to identify and investigate with IPA endpoints and pathways in an explorative way at repeated time points and the data allowed us first information on transcript expression patterns in whole blood from the overwintering crew. Though the analyses did not encompass proteins or metabolites, this approach can allow in the future for more targeted investigations and potential therapies and its monitoring. Hence, we focused on changes present in all subjects across all timepoints compared to the baseline timepoint before isolation (t0). Results evidenced an inhibition of key immune functions relevant for innate and adaptive immunity as well as a downregulation of interferon pathway genes. The changes persisted during isolation and in some cases did not recover after return.

2. Material and methods

2.1. Study design

The data have been collected as part of the CHOICE-II study conducted at the Antarctic *Concordia* station during the winter-over campaign 2017. The study was carried out according to the ethical code of the World Medical Association (Declaration of Helsinki). Ethical approval was obtained from the institutional review board of the Ludwig-Maximilians University (LMU) Munich, Germany, and the medical board of the European Space agency (ESA). All overwintering crew underwent health checks and were considered fit and eligible to participate in this study. The CHOICE II exclusion criteria were smokers and an age of 60 years and above. Eight subjects gave their written informed consent. Although we aimed for a balanced sex distribution, the number of women participating in winter-over campaigns at *Concordia* station is usually low. Therefore, we recruited only male participants with a median and IQR of 36 years (26–44) in age, 1.75m (1.72–1.79) in height and 77 kg (67.00–86.00) in weight with a calculated BMI of 24.37 kg m^{-2} (22.62–27.01). Four participants were Italian, and the four other participants were of French nationality. There were no dropouts and subject privacy rights have been observed at all times. Data sharing is intended, yet legal and data protection rules need to be acknowledged. Due to the small number of participants and openly available information on crew and project, subject identification may be possible. However, data can be provided to external researchers based on confidentiality agreements and on receiving the informed consent from the overwintering subjects for external, new analyses.

2.2. Blood sampling

Blood was sampled by medically trained personnel 2–3 months before deployment (t0), on site at *Concordia* station during January, April, July, October and 5–6 months after the isolation period (post). Timepoints t0 and post were defined and scheduled by ESA and the polar institutes in charge of organizing the mission. During the isolation period, where only one research MD was present, blood draws of all participants were incorporated to the schedules of station work and were executed along the medical board approved protocol at the respective months within a one-week timeframe. 2.5 ml of venous blood was drawn under sterile conditions directly into PAXgene® RNA tubes (BD Biosciences, Germany) and stored at −80 °C according to the manufacturer's recommendation.

2.3. RNA isolation and whole transcriptome sequencing (RNAseq)

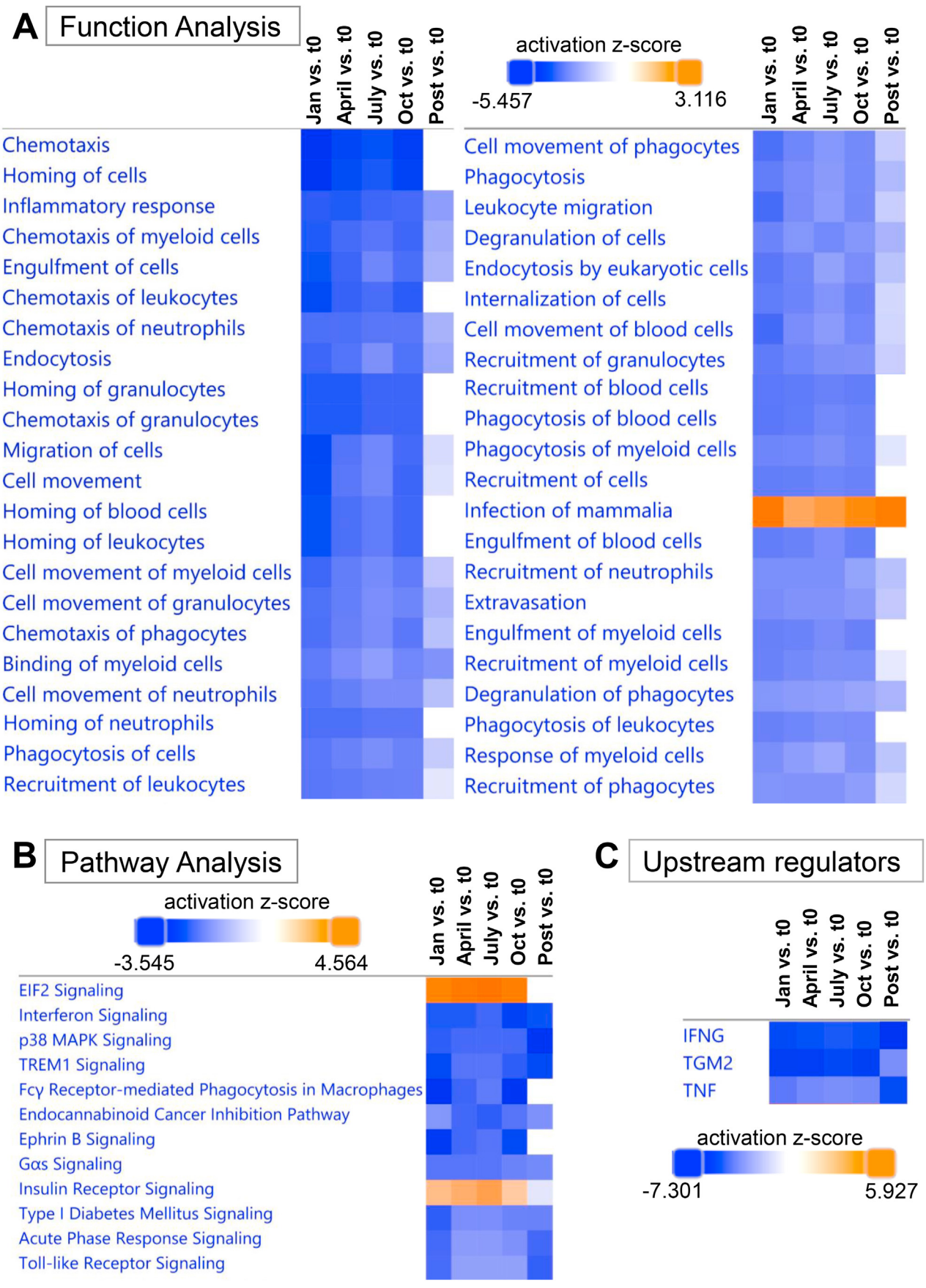
Total RNA isolation was performed from blood samples using the Paxgene Blood RNA Kit (PreAnalytiX GmBH, Germany) according to the manufacturer's instructions including a subsequent purifying step using RNeasy columns (Qiagen, Germany). RNA content and quality were measured using nanoDrop fluorometer (Thermofisher Scientific, Germany). Additional quality controls, random primed cDNA synthesis and RNAseq using next generation sequencing (NGS) was performed at Eurofins® (Konstanz, Germany). NGS was done on an Illumina HiSeq 2500 platform in a paired-end configuration 2 × 150bp with a minimum of 30M reads per sample.

2.4. Sample read preparation and in silico analysis

FASTQ files of RNA sequencing files were imported into the Array Studio software v10.0.1.118 (QIAGEN, Cary, NC, USA) package for further data analysis. All FASTQ files were aligned to the gene model Ensembl. v92 and to the reference library Human B38 using the proprietary OmicSoft Aligner OSA (Hu et al., 2012). Differential gene expression of each timepoint versus t0 was assessed using DESeq2 Love et al., 2014. Differentially expressed genes were sent to IPA (http://www.ingenuity.com) for biological analysis using the cutoffs: q-value $\leq$0.01 (false discovery rate (FDR) applying the Benjamini–Hochberg procedure), fold change (fc) $\geq|2|$and mean counts min $\geq$100 retrieving 2000 genes for analysis. Due to smaller differences between the post isolation and the pre-isolation t0 timepoint the chosen cutoff values for the post isolation timepoint were set to expression p-value: $\leq$0.01, fc $\geq |1.5|$and mean counts of $\geq$10 retrieving 560 genes for analysis.

2.5. IPA statistics and data presentation

IPA statistics is based on two outputs. A p-value derived from a right-tailed Fisher's Exact Test estimates the probability that the association between a function or pathway and a set of molecules might be due to random chance but does not consider directional changes. This



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is, however, predicted for a function, pathway, or upstream regulator (activation or deactivation) by the activation z-score algorithm. The z-score describes the number of standard deviations data lies above or below the mean. A z-score ≥ 2 was considered significantly increased whereas a z-score ≤ -2 was considered significantly decreased (Kramer et al., 2014). We performed a comparison analysis to evaluate transcriptomic changes for Diseases and Functions, Canonical Pathways and Upstream Regulators, a prediction algorithm (Kramer et al., 2014) of IPA.

3. Results

3.1. Biological functions and pathway analysis

The data showed a surprisingly uniform inactivation of cellular processes across all isolation timepoints, except for return (Fig. 1A, data table S1). Most functions indicated a significant impairment of immunity. The functions with the strongest negative z-scores were "chemotaxis" (January (Jan): 5.46 (FDR p value = 4.14E-07), April: 4.71 (p = 4.73E-07), July: 4.41 (p = 4.34E-06), Oct: 4.95 (p = 1.28E-07), post: n. s) and "homing of cells" (Jan: 5.43 (p = 1.28E-07), April: −4.55 (p = 6.66E-08), July: 4.25 (p = 6.26E-07), Oct: 4.86 (p = 4.27E-08), post: n. s). The expression pattern of genes resulting in the strong negative z-score of the function "chemotaxis" (exemplary for the October timepoint versus t0) are displayed in supplemental figure S1and the corresponding fold changes, p-value and q-values and expression intensity are provided intable S2. The inactivation of some functions even persisted after return such as "inflammatory response" (Jan: 4.15 (p = 1.13E-07), April: 4.19 (p = 2.06E-07), July: 3.92 (p = 1.22E-06), Oct: 3.85 (p = 9.37E-08), post: 2.59 (p = 7.74E-08)). Of the few activated functions, "infection of mammalia" showed the strongest positive z-scores (Jan:3.06 (p = 3.16E-07), April:2.08 (p = 1.54E-06), July:2.35 (p = 1.92E-06), Oct:2.64 (p = 1.61E-07), post:2.93 (p = 4.01E-09)) fitting to the overall impression of a dysfunctional immune state.

The predicted activation state of signaling pathways (Fig. 1B, datatable S3), was similar across all timepoints from January to October, though upregulated and downregulated pathways seem balanced compared to functions. The strongest activated pathway was "eukaryotic initiation factor-2 (eIF2) signaling" (mean z-score:4.35 ± 0.20) indicating an increase in genes involved in mRNA translation during exposure to environmental stress (Wek et al., 2006). Furthermore, "Interferon signaling" showed consistently negative z-scores (Jan: 2.714 (p = 4.54E-03), April: 2.714 (p = 1.57E-03), July: 2.53 (p = 2.28E-03), Oct: 3.162 (p = 4.05E-03), post: 2.828 (p = 3.97E-05)) and was the pathway with the second most prominent changes over time. Significantly predicted inhibited canonical pathways across all timepoints included p38-mitogen-activated protein kinase (MAPK, mean z-score: −2.73 ± 0.42) and triggering receptor expressed on myeloid cells 1 (TREM1) signaling (mean z-score: 2.66 ± 0.34) as well as Fcγ receptor-mediated phagocytosis (mean z-score: 2.95 ± 0.65). Consistently, the upstream regulator interferon (IFN) γ is predicted to be inhibited with the strongest negative z-scores, especially after return, (Jan: 6.19 (prediction p-value = 9.44E-08), April: 5.90 (p = 1.31E-07), July: 5.69 (p = 1.34E-07), Oct: 5.88 (p = 4.57E-09), post: 7.30 (p = 3.57E-38)). Other inhibited regulators included transglutaminase 2 (TGM2; Jan: 6.59 (p = 9.15E-16), April: 6.62 (p = 4.79E-15), July: −6.244 (p = 6.65E-13), Oct: 6.54 (p = 9.69E-17), post: 3.97 (p = 1.62E-18)), tumor necrosis factor (TNF; Jan: 4.68 (p = 3.66E-05), April: 4.13 (p = 6.84E-05), July: 3.97 (p = 9.02E-05), Oct: 4.10 (p = 7.14E-06), post: 6.10 (p = 1.00E-18)) (Fig. 1C), and IFNα (mean z-score: 4.20 ± 0.96).

3.2. Analysis of signaling pathways

The gene expression pattern of the pathways IFN (Fig. 2A), p38MAPK (Fig. 2B), TREM1 (Fig. 2C) and eIF2 signaling were analyzed. The majority of genes found upregulated in the eIF2 pathway, were ribosomal proteins (RP) involved in translation (figure S2, corresponding data table S4, representative pathway figure S3A). Among the genes of the IFN pathway 14 genes were downregulated and 2 upregulated across all timepoints (Fig. 2A). The most prominent negative expression fold changes were observed for interferon induced transmembrane protein 2 (IFITM2; Jan: 4.16 (FDR p value = 9.24E-30), April: 4.11 (p = 1.22E-34), July: 4.08 (p = 1.54E-31), Oct: 4.22 (p = 1.40E-30), post: n. s.) and 3 (IFITM3; Jan: 3.75 (p = 1.27E-07), April: 3.69 (p = 9.13E-08), July: 3.73 (p = 1.02E-07), Oct: 3.70 (p = 1.91E-07), post: n. s.) as well as Interferon-α Induced protein with tetratricopeptide repeats 3 (IFIT3). IFIT3 remained downregulated after return; (Jan: 2.98 (p = 2.80E-10), April: 3.04 (p = 1.32E-11), July: 3.07 (p = 2.43E-11), Oct: 3.07 (p = 8.49E-11), post: 2.49 (p = 1.75E-03)). The genes found downregulated across all timepoints in the p38 MAPK (figure S3B) and TREM1 signaling pathways (figure S3C), are involved in the cytokine response such as interleukin 1 receptor (IL1R) and IL1 beta (IL1B) and tumor necrose factor receptor superfamily 1A (TNFRSF1A).

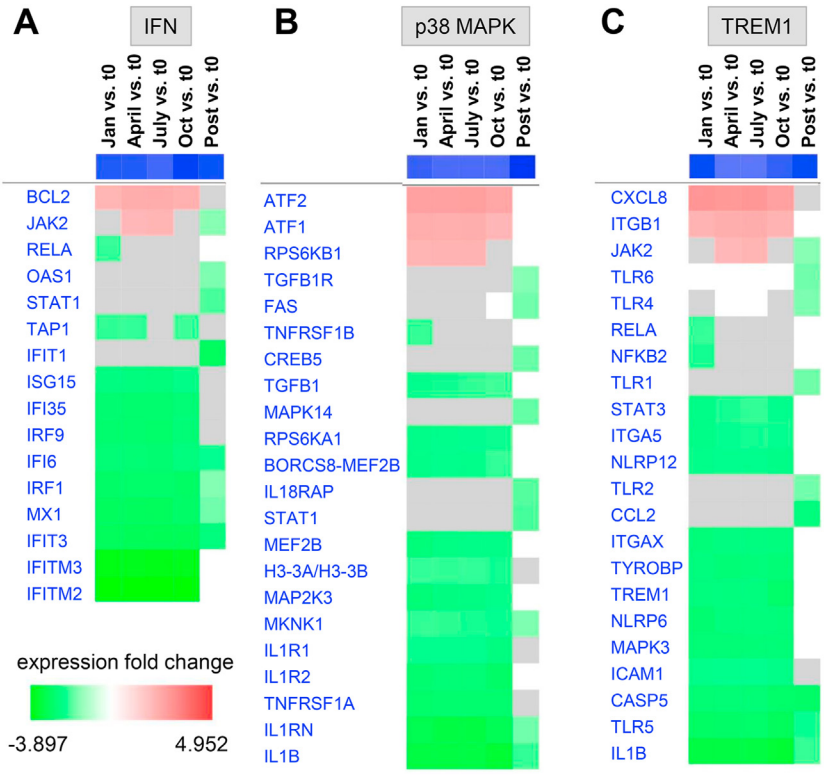
The genes of the IFN pathway that were found downregulated are part of the antiviral immune response as shown for October (Fig. 2D) as example. Multiple IFN stimulated genes (ISG) were downregulated or predicted to be inhibited based on a decreased activity of STAT1. Other timepoints showed a similar pattern (data not shown). The Diseases and Functions prediction algorithm indicated that "antiviral response" was predicted to be decreased and "viral infection" and replication were increased based on the gene expression pattern.

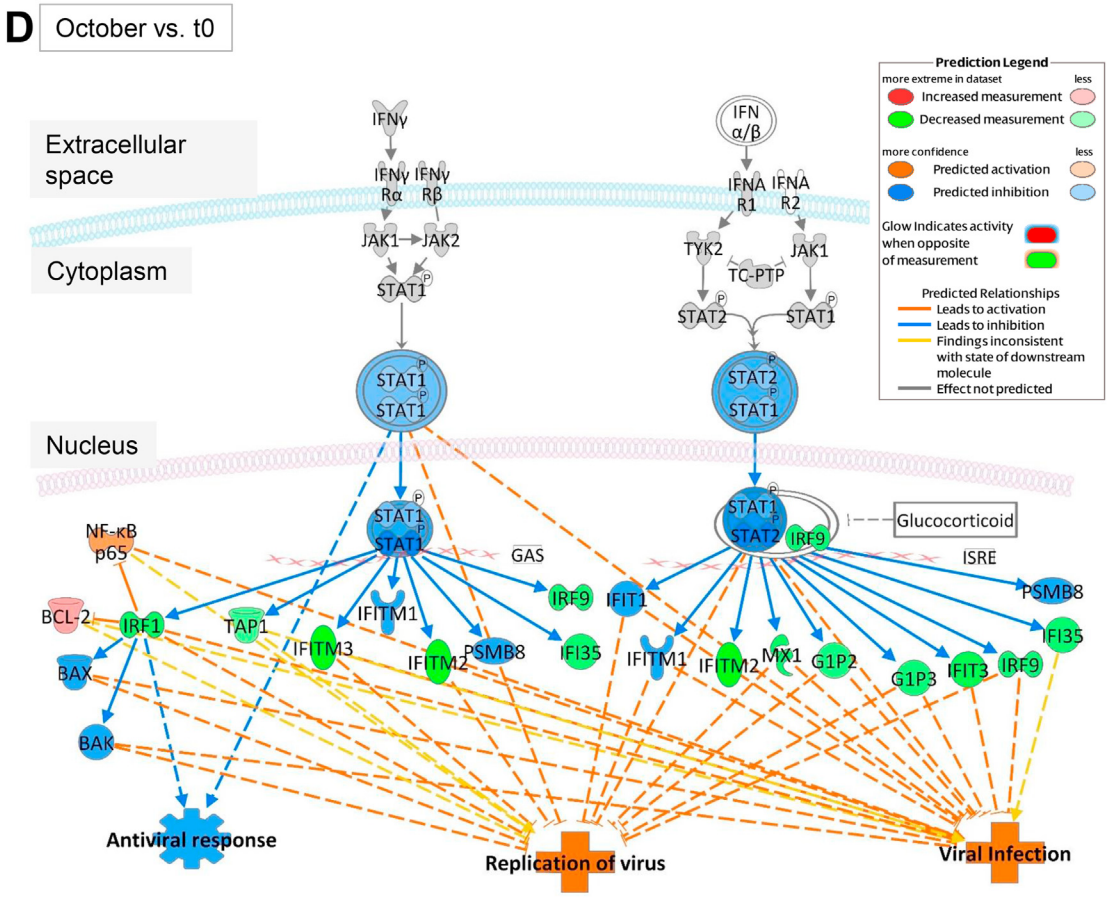
4. Discussion

4.1. Inactivation of key immune functions and pathways

Overwintering in a remote, potentially life-threatening environment puts the human body under an enormous strain. Though the choice to live and work there might support the capability and willingness to cope, the bodily stress response to this high allostatic load is nonetheless detectable (Pagel and Chouker, 2016; Strewe et al., 2018; Sandal et al., 2018). Our results evidenced on the transcriptomic level that the majority of biological functions affected were relevant for a competent immune response and already inactivated at the beginning of isolation in January. Despite undulating expression intensities between timepoints, the overall tendency prevailed until the end of the isolation, or even after return. We did not observe alternating activation states according to the distinct external stressors e.g. the phase of

Fig. 1. Comparison analysis across all timepoints.
(**A**) Color-coded depiction of z-scores (heat-map) showing the inactivation (negative z-score, blue) of most immune functions compared to t0. "Infection of mammalia "was found to be activated (positive z-score, orange) (**B**) Heat-map showing z-scores for the most affected pathways. eIF2 and interferon signaling showed pronounced z-scores reflected in color intensity. (**C**) Heat-map showing the top three upstream regulators: *Interferon* (*IFN*), *transglutaminase 2* (*TGM2*) and *tumor necrosis factor* (*TNF*) that showed negative z-scores (inactivation). (n = 8, male, color intensity reflects z-score. *eIF2: elongation Initiation factor 2, MAPK = Mitogen activated protein kinase, TREM1: The triggering receptor expressed on myeloid cells 1)*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)





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complete darkness in July. While the profound psychosocial effects of the Antarctic winter, characterized by cognition impairment, tension, or conflict between team members, are known to be mission phase dependent, seasonal and to aggravate over time (the "third quarter phenomenon") (Palinkas and Suedfeld, 2008), the overall immune changes on the transcriptomic level seem not to follow this pattern. However, more subtle changes between timepoints may only be visible in functional immune tests (Feuerecker et al., 2019), which were not the focus of this work. Based on our observations, one might assume that adaptation did not occur efficiently or was not completed in the timespan of exposition. During the first week, a stress response is detectable in elevated catecholamine levels together with an inhibition of innate immune functions and higher adenosine blood concentrations due to exposure to hypoxia (Feuerecker et al., 2014). Interestingly, there seem to be limits to fully acclimatize to the chronic hypoxic conditions evidenced by a persistent low oxygen saturation and elevated blood pH (Porcelli et al., 2017). The neuroendocrine stress response follows a similar dynamic, where a tendency for adaptation was detected only at the end of 12-months, which is strikingly different to other coastal research stations (Strewe et al., 2018). Our data tally with those observations and suggest that the same is true for immune signaling.

4.2. Expression pattern suggests vulnerability for viral infections

We detected a strong activation of the eIF2 signaling pathway combined with the inactivation of key immune pathways IFN, p38 MAPK and TREM1 signaling. Since IFN was the top upstream regulator and predicted to be inhibited, we explored genes downstream. Of the 14 genes downregulated across all timepoints, *IFITM* and *IFIT* showed the strongest negative expression fold changes. Both have important antiviral properties (Diamond and Farzan, 2013). IFITM proteins mediate restriction during virus fusion with host-cell membranes e.g. in Severe acute respiratory syndrome (SARS) Coronavirus, Influenza A or Cytomegalovirus (Huang et al., 2011; Ashley et al., 2019). Moreover, *ifitm2* and *3* are expressed by murine T cells and regulate $CD4^+$ helper cell differentiation (Yanez et al., 2020). IFIT proteins exert their antiviral properties through direct binding of non-methylated (non-self) mRNA thereby outcompeting eIF and thus inhibiting the translation process (Fleith et al., 2018). eIF2 is an important mediator of mRNA translation initiation at the ribosome and is an integral element of the cellular stress response (Zhou et al., 2008; Teske et al., 2011). Interestingly, we found eIF2 signaling to be strongly activated. dsRNA >40bp e.g. from viruses can directly activate the interferon-inducible dsRNA-dependent protein kinase (PKR), usually expressed at low levels and inactive, which then phosphorylates the α-subunit of eIF2 (eIF2α) leading to the inhibition of translation of viral RNA (Jackson et al., 2010). We did not observe expression changes of PKR over the time course of the isolation and thus did not investigate the phosphorylation state of both eIF2α and PKR proteins which should be an area of future study. Nevertheless, the combination of an integrated cellular stress response activating translation together with the downregulation of genes critical for an effective antiviral defence identify a possible mechanism by which subjects can become vulnerable to viral infections.

5. Limitations

It was not feasible to compare for sex differences since at *Concordia* the number of women taking part in winter-over campaigns is too low. Work from our own group comparing sex specific differences in winter-over crews at coastal station *NeumayerIII*, showed that women exhibit a slightly different stress response (Strewe et al., 2019). It would therefore be interesting to compare our results to the transcriptome of women at *Concordia* in the future, to extend RNA-seq analyses to other Antarctic stations and to elucidate the role of epigenetic regulation (e.g. via miRNAs) on the genes reported here. The development of viral (or other) infections after the isolation period should be addressed in the future because a follow-up of each individual was not possible at that time, but would be recommended for upcoming studies in order to better support the theory on a higher vulnerability to infection as proposed by the data sets and interpretation. The use of anti-inflammatory, antibiotics or anti-viral drugs during the course of the experiment was not monitored in detail (e.g. by a logbook) and these medical records remain restricted and non-accessible. The regular use of such drugs would have represented a bias in the results. However, blood samples were only taken from subjects when they were healthy and drug use was noted at the time point of blood collection. There was no report about such regular use as self-reported by the crew. Also, if a subject had been ill at a scheduled study time point the corresponding blood sampling would have been postponed until at least 7 days after a complete remission.

6. Conclusion

Taken together, our data show a dysfunctional immune state together with a strong activation of the stress response pathway eIF2. These results suggest a vulnerability to viral infections due to the inactivation of multiple downstream ISG. The majority of changes were present already at the beginning and in some cases did not recover. This indicates that a complete adaptation to this stressful environment does not occur and recovery takes longer than expected that may render winter-over crew potentially more vulnerable for infections also after return. The experience gathered from human space exploration and analog research can help to generate awareness for the prolonged effects of isolation and confinement on physiological systems such as immunity. In conclusion, we might speculate that quarantine measures, such as at *Concordia*, pose people at risk for viral reactivations even well after return which needs further follow-up investigations in the future.

Fig. 2. Detailed Pathway analysis.
Heat-maps showing expression fold changes of genes, which were upregulated (red) or downregulated (green) of the IFN pathway (**A**), p38MAPK (**B**), TREM1 (**C**). Non-significant changes are in grey. Color intensity reflects magnitude of expression fold change. Above the maps are the negative z-scores calculated across all timepoints. Exemplary IFN pathway (October) showing downregulated (green), upregulated (red) or non-differentially expressed (grey) genes. Molecule activity prediction analysis shows genes and biological functions predicted to be inactivated (blue) or activated (orange) according to the expression pattern. Arrows with tipped end (→) show increased effects, arrows with a vertical line at the end (—|) reflect inhibited effects, dashed arrows show indirect effects, straight arrows direct effects, (legend on the top right). Parameters: fold change $\geq |2|$, q-value (FDR) < 0.01. (*IFNR: Interferon receptor, BCL2 apoptosis regulator, JAK1/2: Janus kinase 1/2, RELA: RELA proto-oncogene, OAS1: 2′-5′-oligoadenylate synthetase 1, STAT1: signal transducer and activator of transcription 1, TAP1: transporter 1 ATP binding cassette subfamily B member, ISG15: ISG15 ubiquitin like modifier, IFI35: interferon induced protein 35, IFI6: interferon alpha inducible protein 6, IRF1/9: interferon regulatory factor 1/9, MX1: MX dynamin like GTPase 1, IFIT1/3: Interferon-α Induced protein with tetratricopeptide repeats 1/3, IFITM2/3: interferon induced transmembrane protein 2/3, ISRE: IFN-stimulated regulatory elements, GAS: IFN-activated sequence)*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Funding

This work was supported by the European Space Agency (ESA ELIPS 3 and 4 programs) and the German National Space Program (DLR) on behalf of the German Ministry of Economics and Energy (BWMi) (grants WB1319 and WB1622). IPA Analyses were supported under a "Collaborative Research Agreement" between QIAGEN REDWOOD CITY, INC., USA and the LMU Hospital Munich, though no funding or other financial support was granted by QIAGEN.

Declaration of competing interest

None.

Acknowledgements

We express our thanks to the *Concordia* crew who have participated with great professionalism and all operators, scientists and administrators at the French (IPEV) and Italian (PNRA) polar institutes, NASA, ESA and DLR who made this project possible. We also like to thank Dr. Dominique Moser, Katharina Biere and Marion Hörl at the Laboratory of Translational Research, LMU Munich for their excellent support and technical assistance, respectively.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2020.100145.

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