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Immune sensitization during one year in the Antarctic high altitude Concordia Environment

Short title: Immune-alterations in Antarctica

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Abstract

Background: Antarctica is a challenging environment for humans. It serves as a spaceflight ground-analog, reflecting some conditions of long-duration exploration class space missions. The French-Italian *Concordia* Station in interior Antarctica is a high fidelity analogue, located 1000 km from the coast, at an altitude of 3232m. The aim of this field study was to characterize the extent, dynamics and key mechanisms of the immune adaptation in humans overwintering at *Concordia* for one year.

Methods: This study assessed immune functions in fourteen crewmembers. Quantitative and phenotypic analyses from human blood were performed using on-site flow cytometry together with specific tests on receptor-dependent and independent functional innate and adaptive immune responses. Transcriptome analyses and quantitative identification of key response genes were assessed.

Results: Dynamic immune activation and a two-step escalation/activation pattern were observed. The early phase was characterized by moderately sensitized global immune responses, while after 3-4 months immune responses were highly up-regulated. The cytokine responses to an *ex vivo* stimulation were markedly raised above baseline levels. These functional observations were reflected at the gene transcriptional level in particular through the modulation of hypoxia-driven pathways.

Conclusions: This study revealed unique in-sights into the extent, dynamics and genetics of immune dysfunctions in humans exposed for one year to the Antarctic environment at the

Concordia station. The scale of immune function was imbalanced towards a sensitizing of inflammatory pathways.

Highlights

- Immune sensitization during one year at high altitude Antarctica
- Unexpectedly alterations of the purinergic and HIF-1 α pathways
- On-site flow cytometric cell measurements at high altitude Antarctica

Background

ANTARCTICA - though it is the fifth largest continent with a size of more than 14 million km² (1.3 times of the surface of Europe) - it is the least populated (1). During winter, an average 1,100 people live at different research stations, whilst during the summer months the population increases up to 5000 habitants. Some of the research stations are only accessible during the summer seasons where 24h sunlight and more moderate temperatures allow access (2). In contrast, during winter, the constant winter darkness limits rescue and evacuation possibilities. Of all the Antarctic bases, the French-Italian *Concordia* Station, located at Dome C (75° 06' S - 123° 21' E) is considered one of the harshest stations to winter over at. Whilst most Antarctic bases are located along the coast, *Concordia* is located 1000km from the coast, at an altitude of 3232m, leading to a calculated fractional inspiratory oxygen fraction of 13% and temperatures as low as -85°C. The terrain is flat and white, without vegetation or wildlife. There are 3 months of constant sunlight during the summer, and 3 months of constant darkness during the winter. Crew sizes are between 10 and 16

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members of mainly French and Italian nationalities. There is no resupply during the 9-month winter period. Between 2008 and 2011, there was no internet access, and telecommunications consisted of email ‘dumps’ twice a day and satellite telephone call.

Due to this unique setting, the *Concordia* Station is seen by the European Space Agency (ESA) as an ideal platform to investigate several conditions which will be found in future space missions and in lunar or Martian habitats. These conditions include social isolation, confinement, altered day/night cycles, lower atmospheric oxygen concentrations, and extremes of temperature.

(http://www.esa.int/Our_Activities/Human_Spaceflight/Concordia/The_remotest_base_on_Earth)

The goal of this field investigation was to use *Concordia* to assess the degree and dynamics of innate and adaptive immune changes in humans resulting from long-term isolation and confinement in a hypobaric hypoxic setting. The use of a specifically designed battery of tests for on-site immune monitoring as well as semi-quantitative and quantitative transcript analyses allowed targeting for questions also relevant for long-term space flight. The data also helps to provide a translational understanding of the effects of chronic hypoxia on humans to explore more clinically relevant conditions and pathologies.

Methods

Study design and protocol

The winter-over isolation period lasted for 9 months, whilst the summer ‘transition’ period lasted for 3 months. Subjects were in Antarctica for approximately one year. Following

solicitation, screening and enrolment, 6 subjects participated in the 2009 winterover period, and 9 subjects participated during the 2010 season (Supplemental Table 1, Supplemental material).

Sample schedule and collection

Baseline data collection (Baseline Europe) took place in Paris, France or at the European Space Research and Technology Centre (ESTEC), Noordwijk, Netherlands approximately 2 months prior to departure to Antarctica. At *Concordia*, blood samples were collected for the test-battery at four different time points throughout the overwintering period (“**3-4weeks**”: December/January, “**4months**”: March/April, “**7months**”: June/July, “**11months**”: October/November, Supplemental Table 2). After an overnight fasting period, blood was collected into heparinized and EDTA anticoagulated tubes. Due to the first-time deployment of a flow cytometer to *Concordia* Station, peripheral blood phenotyping and functional analyses with live cells could be performed. Other blood sample aliquots were also processed at *Concordia* Station, including plasma separation and short-term cell cultures. Some samples were then stabilized in conditioned storage for transport to Europe/USA after the winterover. Samples were only taken from subjects when they were healthy. If a subject was ill at the scheduled study time point, sampling was postponed until at least 1 week after complete remission. During the two observed years only one time point in one individual had to be postponed. This happened in September 2009 and the original blood draw was shifted by two weeks.

A Blood cell count, differentiation, and circulating inflammatory markers

1. Complete blood count (CBC)

CBCs were performed from EDTA-anti-coagulated blood samples using the on-site QBC-Autoread-plus automated analyzing system (QBC-Diagnostics, Port Matilda, PA, USA). Hematocrit, hemoglobin concentration, platelet and differential white blood cell counts, as well as the percentages of granulocytes and lymphocytes were quantified.

2. Differential blood count/ Peripheral leukocyte distribution/ Immunophenotype analyses

A three-color flow cytometry antibody based strategy for immunophenotype analysis was designed to assess all major leukocyte, lymphocyte and T-cell subsets. The fluorochromes were FITC, PE and PE-CY5, and were compatible with the Partec flow cytometers' excitation/emission characteristics (Cyflow SL®, Partec, Münster, Germany). Blood sample staining, lysis, and flow cytometry analyses were set up and performed as previously described (3). The bulk leukocyte or lymphocyte populations were identified by forward and side scatter properties. Sequential gating by immunoscatter was employed as appropriate, such that relative percentages were derived from a positively-identified parent population. For example, from a lymphocyte gate, T-cells were identified and gated from a plot of CD3+, and both CD4+ and CD8+ were plotted from the T-cell gate (see Supplemental Table 3).

3. Measurement of Plasma Cytokine Concentrations

The concentrations for 22 plasma cytokines representing five categories of function (inflammatory, T-regulatory Th1/Th2, chemokines, growth factors) were determined

simultaneously in duplicate using a commercially available multiplex bead immunoassay (R&D Systems, Minneapolis, MN, USA). EDTA anticoagulated blood samples were immediately centrifuged and supernatants were preserved frozen (-60°C) until measurements in the authors' labs using a Luminex 100 instrument (Luminex, Inc., Austin, TX, USA). All samples were measured within a time frame of 2 years after sample collection. De Jager et al. (4) were able to show that frozen cytokines remain stable up to 2 years.

B Immune cell functions

In vitro functional innate immunity

1. Granulocytotic/ polymorphonuclear leukocytes (PMN) oxidative burst

Leukocytes were isolated by Ficoll-Histopaque (Sigma-Aldrich, Steinheim, Germany) gradient sedimentation to obtain leukocyte enriched plasma (LRP) containing $\sim 1 \times 10^5$ leukocytes (PMN from polymorphonuclear neutrophils) after sedimentation of erythrocytes. LRP was incubated with Dihydrorhodamine (10^{-6} mol/L) in 0.5 ml HBSS. LRP was stimulated by either tumor necrosis factor (TNF; 10 ng/ml) and N-formyl-methionylleucyl-phenylalanine (fMLP, 10^{-7} M) or phorbol myristate acetate (PMA; 10^{-9} M). After 30 minutes of stimulation, cell activation was stopped by placing tubes on ice. Production of H_2O_2 by PMNs was determined by onsite flow cytometry with specific gating on the PMNs (Forward/Sideward scatter) (2). The effects of stimulation were expressed as percentage towards the negative control with no stimulation.

Leukocyte enriched plasma was also incubated prior to activation with TNF and fMLP with increasing concentrations of adenosine (10^{-9} to 10^{-5} mol/L) to assess the adenosine receptor-dependent inhibition curve and sensitivity to the immune modulatory adenosine (5). After termination of the activation, production of H_2O_2 was analyzed by flow cytometry, as before. (Further granulocyte test in Supplemental material/Supplemental Figure 2)

2. Monocyte/LPS stimulation assay

For analysis of secreted cytokine profiles after LPS stimulation, whole blood culture was performed by combining 150 μ l of heparinized blood and 1.0ml RPMI-media. Culture was performed using 10 μ g/ml LPS E.-coli serotype 026:B6 (Sigma-Aldrich) for monocyte activation and was incubated for 48hours at 37°C. Following culture, supernatants (media with diluted plasma) were removed and frozen until analysis. Here, an inflammatory bead array assay (Becton Dickinson, Miami, Florida, USA) was subsequently used for TNF, IL-1 β and IL-10 quantification. For analysis, a Beckman-Coulter Gallios' flow cytometer was configured to resolve all six bead populations and allow increases in cytokine concentration to be detected. Since all subject samples were batch-analyzed, data were recorded as mean fluorescence intensity (MFI) and subsequently converted to pg/ml concentration, to show subject relative cytokine production alterations throughout the Antarctica deployment.

3. *Evaluation of immunocyte functional capacity/ cytokine production following mitogenic stimulation*

24 hours culture

T-cell function (early blastogenesis): T-cells were activated during whole blood culture (24 hours, 37°C) in the presence of antibodies which trigger TCR-activation (anti-CD3) and provide co-stimulation (anti-CD28). Culture was performed by combining 100µl of heparinized blood, 1.0ml RPMI media, 10µl each of anti-CD3 and anti-CD28 (Becton Dickinson, Franklin Lakes, NJ, USA) or 10µg each of Staphylococcus enterotoxin A (SEA) and B (SEB) (Sigma-Aldrich, St Louis, MO, USA). Following culture, T-cell progression through a full activation cycle was monitored by determining the expression of CD69 and CD25. CD69 exists pre-formed in the cytoplasm and is rapidly expressed ~1 hour after activation, whereas CD25 requires new gene transcription and protein synthesis and is expressed after 18-24 hours of activation. A four-color staining of cell surface markers (CD25/FITC, CD69/PE, CD4 or 8/PC5) was performed as described above. The gating strategy consisted of T cell resolution and separation into CD4 and CD8 subsets followed by enumeration of both, total CD69+ and CD69+/CD25+ dual positive events. This assay was only performed during the early and late deployment phases when a visiting scientist from LMU Munich or NASA was present.

48 hours culture

All assay tubes contained DMEM as media and either: no stimulus (spontaneous cytokine release); or a bacterial antigen mixture containing diphtheria-, tetanus- and pertussis-toxoid

(all three combined in 1% Boostrix®; GlaxoSmithKline, Munich, Germany); or a fungal antigen mixture containing *Candida* lysate (10mg/mL; Allergopharma, Reinbeck, Germany) and *Trichophyton* lysate (10mg/mL; Allergopharma, Reinbeck, Germany). Further, pokeweed mitogen (PWM) (5mg/mL; Sigma-Aldrich, Steinheim, Germany), a strong polyclonal activator that induces mitosis in lymphocytes in a non-receptor-specific manner, was added as stimulus to one assay tube. Additional stimulations were done in the presence of 0.125µg/mL anti-CD3 and 0.25µg/mL anti-CD28 (Becton Dickinson, Franklin Lakes, NJ) to activate T-cells only via the T-cell receptor with co-stimulation; or 100ng/mL PMA + 2µg/mL ionomycin (both Sigma-Aldrich, St. Louis, MO) as a protein kinase C activator and pan-specific B-cell and T-cell activator. 400µL whole lithium heparinized blood was added to the assay tubes and subsequently incubated for 48h at 37°C on-site. Total tube volume was 800µL. Thereafter, supernatant was transferred into Eppendorf tubes and frozen immediately at -60°C for future cytokine analyses (methods as described in supplemental material).

C Genetic/transcriptomic analyses

RNA extraction, microarray preparation and analysis

Microarray analyses were performed on leukocytes that were isolated from EDTA blood samples (3ml) using LeukoLOCK™ Total RNA Isolation Kit according to the manufacturer's instructions (Applied Biosystems Foster City, CA, USA). After total RNA extraction, RNA quantity and quality were assessed using a Nanodrop 2000c spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) and a 2100 BioAnalyzer (Agilent, Santa Clara, CA, USA). All samples were of sufficient quality for further processing. Briefly, 150ng of total

RNA was used to prepare complementary DNA using the Ambion WT Expression Kit (Affymetrix, Santa Clara, CA, USA). Complementary DNA was in vitro transcribed to biotin labeled complementary RNA, which was fragmented and hybridized for 16 h at 45°C onto Affymetrix Human Gene 2.0 ST arrays. Arrays were washed and stained using the GeneChip Hybridization, Wash and Stain kit (Stain Module) and scanned using an Affymetrix GeneChip Scanner 3000 7G scanner.

CEL-files were imported into Partek Genomics Suite v6.6. Exon-level data normalization was performed using a customized Robust Multi-array Average algorithm (background correction for entire probe sequence, quantile normalization, log₂ transformation of signal intensity values). Gene level data were obtained by summarizing exon level signals using Tukey's biweight algorithm. This generated a total of 44,629 probe sets corresponding to a gene. Of these, 17,247 (39%) had no annotation, the majority of which were likely to target non-coding RNA genes.

Quantitative reverse transcriptase PCR (qRT-PCR)

Complementary DNA was prepared from 2µg of total RNA using the GoScript Reverse Transcriptase kit (Promega, Leiden, The Netherlands) using 1µL of random hexamer primers and 3.75mM MgCl₂ in 20µL reactions (RNA obtained from leukocytes collected with LeukoLOCK™). Taqman primer assays for each gene (Supplemental Table 4) were used according to the manufacturer's instructions in a 7500 Fast real-time PCR instrument (Applied Biosystems). Relative quantification of mRNA expression was performed according to the $\Delta\Delta C_t$ method. For normalization, we used the geometric mean of Ct-values of three reference genes (TBP, 18S and RPLP0).

D Statistical Analysis

Data analysis was performed with commercially available software (SPSS 20.0; IBM and SigmaPlot 12.5; Systat). Normal distribution was tested by the Shapiro-Wilk test. Within group comparisons were made for normally distributed data by a one way repeated measures analysis of variances (one way RM-ANOVA) followed by the post-hoc Holm-Sidak method. Not normally distributed data were analyzed by a one-way ANOVA on ranks followed by Dunn's test. This was a longitudinal study where all deployed data were compared to a single baseline measurement. Data are considered to be significant at $p < 0.05$ and are indicated as such (*, bold) on each data table and figure. To enable multiple testing in the case of missing data in data sets but with an $n \geq 12$, data were imputed by simple imputation of the mean value of that parameters' time points. As simple data imputation can only be done when 1 or 2 data sets are missing (6), data imputation was only performed in a few cases. If n was below 12, no data imputation was applied. Results are expressed as means $\pm$ SD. To increase clarity, data displayed in figures are expressed as means $\pm$ SEM.

Results

A Blood cell count, differentiation, and circulating inflammatory markers

A standard battery of blood cell count, cell differentiation and plasma inflammation markers was performed to obtain information if the *Concordia* environment provokes cellular changes or systemic inflammation:

1. Complete blood count

Hemoglobin-concentration, hematocrit, thrombocyte and lymphocyte counts increased significantly throughout the stay at *Concordia*. Granulocytes and monocytes showed a

significant elevation after “4months”and “7months” before returning to baseline values (Table 1).

2. *Differential blood count/Peripheral leukocyte distribution*

Peripheral leukocyte distribution

During the overwinter period, there was a significant decrease among lymphocyte subsets in the T-cell percentage, with a corresponding increase in B-cells. Number of Natural Killer (NK) cells was also elevated, except for time point “7months”. Differentiation of T-cells revealed that the decrease was within both subsets, CD4+ and CD8+. However, percentages of ‘bulk memory’ T-cells increased during the overwinter period. Naïve cytotoxic CD8+ cells significantly decreased, whereas the senescent population significantly increased during deployment, as did central memory cells. Also the percentage of terminally differentiated T-cells increased, reaching the level of statistical significance at the second half of the winterover, while both populations of activated T-cells (CD69+ or HLA-DR) showed a significant peak during the first month of deployment (3-4weeks, Table 2) only .

3. *Plasma Cytokine Concentrations*

Compared to baseline, there was little deviation in the concentration of most of the pro- and anti-inflammatory cytokines and chemokines (Supplemental Table 5). A few plasma cytokines changed consistently, although not always reaching statistical significance. There was a consistent overwinter decrease in the concentrations of IL-1RA, the receptor antagonist to Interleukin 1, while the concentrations of granulocyte colony stimulating

factor, G-CSF, and epithelial-derived neutrophil-activating peptide (ENA-78) significantly increased (Table 3). Incubation of whole blood on media (DMEM) without stimuli for 48 hours revealed a significant increase in the spontaneous production of TNF during the winterover (Table 3).

B Immune cell functions

A panel of *ex vivo* immune stimulation assays were performed to determine the functional capacities of the innate (1. granulocytotoxic oxidative burst and inhibition, 2. monocytotoxic function (LPS stimulation)) and adaptive (3. re-call antigen or mitogen stimulation) immunity.

In vitro functional innate immunity

1. Granulocytotoxic/ polymorphonuclear leukocytes (PMN) oxidative burst

The H₂O₂ production-rate of PMN upon receptor-dependent stimulation with TNF/fMLP or with the non-receptor dependent protein kinase C activator PMA showed a typical stimulus-dependent response. The stronger stimulus PMA resulted in higher H₂O₂ rates. With either stimulation, the maximum responses emerged during the deep winter phase with a statistically significant peak after PMA stimulation (7months) (Figure 1).

In vitro modulation of TNF/fMLP-stimulated H₂O₂ production by adenosine

Increasing adenosine concentrations dose-dependently inhibited the TNF/fMLP induced H₂O₂ production by PMN. The adenosine dose-response curves showed a sigmoidal shape. The maximal inhibitory adenosine effect (10⁻⁵M) was observed during deep winter (7months) (Supplemental Figure 1). The half-maximal inhibitory concentrations (IC₅₀)

increased from 52.1nM (Baseline Europe) to the maximum during winterover to 86 nM (7months).

2. *LPS stimulation assay*

Following whole blood stimulation with LPS, TNF and IL-10 increased significantly during deployment, followed by a decrease after “11months” at *Concordia*. IL-1 β showed an early (not significant, n.s.) increase after “3-4weeks” followed by a return to baseline values before a significant elevation occurred after “11months” (Table 3).

3. *Cellular function following re-call antigen or mitogen stimulation*

Stimulation with bacterial and fungal antigen mixtures revealed a significant increase for the pro-inflammatory marker cytokines TNF, IFN- γ and IL-2. Cytokine levels were highest during deep winter (7months, see Figure 2 A/B/C). The B- and T-cell responses upon stimulation with PWM followed similar kinetics. The increase in the production of pro-inflammatory cytokines was paralleled by an increase in the anti-inflammatory cytokine IL-10 (Figure 2D). Following PMA and ionomycin stimulation, fairly consistent overwinter elevations were observed in the production of TNF (**TNF**: Baseline Europe : 1311.6 ± 654.1 ; 3-4weeks: 2346.5 ± 1507.7 ; 4months: $4041.1 \pm 2949.2^*$; 7months: 2835.2 ± 1403.9 ; 11months: 865.8 ± 717.6 ; [pg/ml], n=12-14; * p<0.05 vs. Baseline Europe) and IL-10 (**IL-10**: Baseline Europe : 101.4 ± 78.2 ; 3-4weeks: $389.2 \pm 194.5^*$; 4months: $430.6 \pm 190.1^*$; 7months: $353.7 \pm 241.4^*$; 11months: 84.8 ± 79.8 ; [pg/ml], n=12-14, *p<0.05 vs. Baseline Europe).

T-cell function/early blastogenesis: Compared to baseline, T-cell function was elevated during the earlier adaptation phase of deployment, though only in 4-6 participants (Supplemental Table 6). During the late phase (11months), stimulation with SEA+SEB also yielded increased T-cell function (total CD69+ cells only). CD3/CD28 responses were normal during late deployment.

C Gene expression analyses

To obtain insight in underlying pathways influencing immunity, such as e.g. hypoxic pathways, we performed microarray analyses and confirmative qPCRs.

Microarray data revealed a similar pattern regarding the overall gene expression profiles. Unsupervised hierarchical clustering and principal component analysis showed essentially three main clusters of samples, namely Baseline Europe, “3-4weeks” and “4, 7, 11months” at *Concordia* (Supplemental Figure 3).

Selected statistically significant changes in the microarray data were confirmed by quantitative PCR. The T-cell co-stimulant receptors CD28 and CD80, acting upon stimulation as an inductor for T-cell proliferation and subsequent cytokine production, were significantly downregulated when compared “3-4weeks” with “11months”. B- and T-lymphocyte attenuator (BTLA) was increased at “3-4weeks” vs. Baseline Europe, but was then significantly downregulated during the winterover (3-4weeks vs. 7months and vs. 11months) (Figure 3A). Signaling lymphocytic activation molecule family 1 (SLAMF-1) mRNA was early increased (significant Baseline vs. 3-4weeks) and remained elevated before a significant decrease (3-4weeks vs. 11months). Altogether, expression of these four genes was significantly reduced after “11months” in comparison to “3-4weeks”.

The hypoxia-sensitive and known immune-directed modulatory signaling pathways as identified by the gene array were also confirmed by qPCR: adenosine deaminase (ADA) decreased significantly from Baseline Europe to “4, 7 and 11months”. Adenosine receptor 1 and 3 were not affected, while the expression of the adenosine receptor 2 subtype (ADORA2B) expression was significantly lower after “3-4weeks, 4months and 7months” (vs. Baseline Europe). Hypoxia inducible factor 1 alpha (HIF1- α) was significantly diminished after “4months” (vs. Baseline Europe, Figure 3B). Throughout the deployment vascular endothelial growth factor A (VEGF-A) mRNA was significantly less expressed compared to baseline. Interleukin Adhesion Molecule 1 (ICAM-1), a factor known to be expressed on macrophages and lymphocytes and which stabilizes cell-to-cell adhesion and activations, was significantly reduced during earlier winterover (3-4weeks and 4months).

Discussion

Exposure of healthy humans to a yearlong isolation and confinement in inner Antarctica at an altitude of ~3200m induced a state of differentiated immune activation. Innate and adaptive functional and transcriptomic response patterns reveal a two-step “escalation” pathway. The immune system in the early phase (3-4weeks at *Concordia*) was moderately sensitized, in contrast to the following months with further enhanced immune sensitization (as summarized in the Graphical Abstract/Figure 4).

When activated with various stimuli, the innate and the adaptive immune responses were sensitized and up-regulated when re-exposed to recall-antigen and mitogen stimulation in vitro. Adaptive immune functions also tended to shift towards an altered T-cell response paralleled with an accentuated B-cell response. This was accompanied by changes in

hypoxia-driven pathways as well as notably less effective and less sensitive adenosine-related immune control. The scale of immune function and quantitative gene expressions of key regulators, enzymes and receptors, indicates a priming towards “(hyper)-inflammation” by diminishing inhibitory pathways and therefore sensitizing inflammatory pathways.

Blood cells and inflammation markers

Enumerative and phenotyping clusters revealed typical high altitude-related changes (7, 8), but also new and unexpected alterations. Aside from hemoconcentration during the entire Antarctic observation period, thrombocytosis was observed. Chronic hypoxia impacts megakaryocytes provoking their proliferation and differentiation (9, 10). These findings imply an increased metabolism of the bone marrow with a possible effect on other cell populations.

The phenotypic analysis of lymphocyte subsets showed a significant increase in the B-cell percentage during the deep winter period (7months), mirrored by a decrease in the T-cell percentage. Among CD8⁺ T-cell subsets, there was a right shift towards a more mature phenotype, as indicated by a decrease in naïve/cytotoxic CD8⁺ T-cells (CD28⁺/C1.7^{+/-}) and increase in the senescent population (CD28⁻) during the winterover period. An increase in constitutively activated T-cells (expressing CD69 or HLA-DR), thought to correlate with in vivo immune activation, was observed only after “3-4 weeks” during the early phase for both winterover groups. As this period consists of dozens of new arrivals at *Concordia*, all undergoing the early adaptation to the environment, such an increase is not unexpected. This is in accordance with the observed endemic infections during the summer campaigns. During the first observed summer campaign 44 people suffered from an infection. In total,

62 cases of infections were reported to the station doctor: 29 people had flu symptoms (influenza like, no influenza testing available at *Concordia*), 18 had upper respiratory tract infections, 10 had gastroenteritis, 3 had a bladder infection and 2 had otitis. 52 people in the second summer campaign showed symptoms of an infection (28 sore throats, 8 rhinitis, 7 tonsillitis, 7 bronchitis, 2 cold symptoms). These clinical observations correlate with the elevation in constitutively activated T-cells for the study participants, even though at the time point of sample collection none of the participants showed signs of infections.

From the assessment of 22 different plasma cytokine concentrations, expressing the unstimulated immune condition, we identified a modest immune activation during the winterover, with a reduction in IL-1RA levels and a significant increase of G-CSF and ENA-78 which are promoting the neutrophil homeostasis (11). These findings are partially opposite to previously described data during Antarctica deployment, including a 2-month inland traverse (12). While Shirai et al. (12) as well as Shearer et al. (13) also described a decrease in IL-1RA concentrations, Shirai found significant reductions in the pro-inflammatory cytokines TNF, IL-6 and IL-1 β during deployment when compared to the baseline data points. However, their baseline values of TNF and IL-6 were above normal clinical values for healthy humans and the decrease observed in Antarctica might reflect a normalization to baseline values rather than a cold or high altitude-related alteration.

Interestingly, Yadav et al. (14) reported a Th1 shifted pattern of cytokines with increased TNF and IFN- γ and decreased TGF- β during winterover. This observed imbalance towards a pro vs. anti-inflammatory cytokine release complements our unstimulated cytokine data and can be mostly ascribed to monocytes (15, 16), which increased in number and percentage during winterover. Besides the moderately activated native status, the incubation of whole blood without a stimulus elucidated intrinsic changes reflecting a state where the Th1 response

was leveraged. This seems to reflect already activated and/or primed conditions which could be then prone to an even more pronounced response upon stimulation.

Immune cell functions after stimulation

Innate immune functional capacities were challenged by stimulating PMNs and monocytes.

An increase in H₂O₂ production by PMNs upon both receptor-dependent and -independent stimulation was observed, peaking during the deep winter phase. Such an elevated H₂O₂ production is a consequence of PMN “priming”, a mechanism to increase sensitivity for a fast response upon stimulation and to facilitate a full activation (17). Primed PMNs exist under diverse inflammatory conditions (18-20), and primed PMNs do not release -upon priming stimulus- their proteolytic granular content (17).

The monocyte production of TNF and IL-10 after 48 hours whole blood culture with LPS was also significantly elevated after “4 months” and “7 months”, whereas IL-1β release was only elevated at the early and late transition period (“3-4 week”, “11 months”). These results also suggest an increased activation status of monocytes and possible priming of immune responses during deep winter. This latter finding may correlate with the early period of endemic illnesses observed at *Concordia*, however a one to one correlation could not be done due to the overall limited cases.

Adaptive immune cell functions

Stimulation by bacterial and fungal re-call antigens resulted in increases in TNF, IFN-γ and IL-2, with peak concentrations at “7 months” for IFN-γ and IL-2 in most assays. IL-10 production peaked for the bacterial antigen challenge during the early phase “3-4 weeks”, whilst fungal

stimulation peaked at the late phase. PWM stimulation resulted in an incrementing increase in pro-inflammatory cytokines (TNF, IFN- γ , IL-2) throughout the time at *Concordia*, but also a significant increase in the anti-inflammatory cytokine IL-10. A similar pattern (TNF and IL-10) was observed for PMA/Ionomycin stimulations. Similarly, direct stimulation of T-cells with CD3/28 also demonstrated increased early and late function via expression of cellular activation antigens and increased cytokine production, which confirms the sensitization of adaptive/T-cell mediated responses.

This pattern of a lymphocytosis and generally heightened B- and T-cell functional responses is somewhat discordant with previous findings on immune function in Antarctica showing decreased cell-mediated immunity, as measured by the skin multitest assay (21, 22). Tingate et al. (23) reported reduced T-cell proliferation in response to the PHA mitogen. However, a major difference between studies in the past at other bases is the presence of long term hypobaric hypoxia at *Concordia*.

Effects of long-term Hypoxia on purine and HIF signaling

Hypoxia strongly affects ATP metabolism triggering purine-mediated immune modulation (24, 25). In models of short term hypoxia, this increase in catabolism of ATP to adenosine results in immune suppression (26). However, granulocyte oxidative burst was less inhibited by adenosine during deep winter phase, suggesting the inhibitory effect of adenosine was less effective leading to a disinhibition of innate immune functions via “weakening” of an inhibitory/immunosuppressive pathway. Gene array analyses and qPCRs confirmed that key elements of the purinergic system were significantly altered and dysregulated, indicating to some extent an adaptive process of disinhibition of purinergic signaling: *ADORA2B* was

significantly downregulated whereas *ADORA1* and *ADORA3* were not affected. These results could be linked to the increased Th1-weighted adaptive immune responses in the described assays since, for example, adenosine A2B receptor activation promotes a type 2 activation of antigen presenting cells, and induction of T_{reg} and myeloid-derived suppressor cells (27). Whether the decreased expression of *ADA* in this regard is a cause or the consequence of downregulated receptors to the ligand adenosine is unknown. However, animal models indicate that low ADA can lead in long term disease models to inflammation which could - with the given limitations of this study - support the enhanced immune responses in our chronic model (28).

The downregulation of the hypoxia inducible factor 1 α (HIF-1 α) during overwintering was unexpected, but could possibly explain some of the observed immune responses. It is known that hypoxia induces inflammation and that the HIF pathway exerts control over inflammatory processes (24). For example, HIF-1 α dependent regulation induces T cells responses shifting from a Th1 to a Th2 cell response (29). HIF-1 α is strongly regulated at a posttranscriptional level. Our data do not automatically entail corresponding changes at the translational level. However, a possible downregulation of transcriptionally active HIF-1 α is suggested by the decrease in VEGF-A mRNA, which is known to be under direct control of HIF-1 α (30). Nevertheless, the downregulation of HIF-1 α expression is unexpected. One explanation could be that our sampling time-points missed the very early phase of adaptation to hypoxia and HIF-1 α up-regulation. A few publications have reported a short-lived transient activation of HIF-1 α dependent pathways in the context of chronic hypoxia in humans. These studies examined the effects of hypoxia in genetically adapted populations e.g. Tibetans and Andeans (31, 32). Petousi et al.(33) reported that "Tibetans living at sea level exhibit a hyporesponsive hypoxia-inducible factor system and blunted physiological

responses upon exposure to hypoxia". The responses in non-adapted subjects may obviously differ. A situation similar to the one represented by our study was described by Goyal et al. (34). They showed no significant differences in protein levels of HIF-1 α in sheep exposed to ~3800m altitude for 110 days. In another animal model, Baze et al. (35) describes an altered gene expression pattern in murine livers subjected to chronic hypoxia. They found that: "after 32 days, the previously described stress pathways, and in particular the HIF pathway, are no longer significant factors in regulating and maintaining physiological acclimation to hypoxia."(35)

Taken together, these examples demonstrate that the hypoxia->HIF-1 α pathway might be activated only transiently in the earlier phase of a chronic exposition to hypoxia.

Effects of long-term Hypoxia on SLAMF and BTLA expression

In addition, qPCR results showed further alterations in receptors relevant for immune cell modulatory pathways. The SLAMF-1 (Signaling lymphocytic activation molecule family 1) receptor was increased at the early time point and remained elevated before returning to baseline at "11months". SLAMF-1 is involved in T-cell development and function (36, 37), for instance by induction of IFN- γ production via an IL-2-independent proliferation of activated T-cells (38). B-cell activation and memory generation, macrophage dendritic cell, neutrophil, and eosinophil function are also regulated by SLAMF-1 receptors. Detre et al. (36) reviewed the involvement of the SLAM family in murine lupus models and observed that several investigations point to their involvement in the control of humoral autoimmunity, primarily via CD4⁺ Th cells. The dysregulation of the immune system at *Concordia* also fits to the decreased expression of BTLA (B- and T-lymphocyte attenuator). Several recent studies demonstrated its critical role in immune regulation in BTLA deficient mice (39-41), which

show a higher susceptibility to inflammation compared to wild-type mice. One of the first reports on BTLA expression in humans suffering from Behcet's disease (BD) was recently done by Ye et al. (42). Ocular BD was associated with a diminished expression of BTLA. The authors found indications of an altered control of pro-inflammatory responses which might be causal to the disease development.

Since such alterations of SLAMF-1 and BTLA expression are significantly involved in hypersensitivity diseases, and in view of the observed disinhibition of the purinergic system and the reduced HIF1- α and VEGFA expression, one might speculate whether such long-term confinement at *Concordia* results in an increased incidence of clinically relevant hypersensitivity reactions, either allergic or autoimmune. It is interesting that after "11months" innate and adaptive immune functions of our overwintering cohorts - although still highly elevated - tend to partially decrease again. This is also mirrored on the transcriptomic levels of the T-cell proliferation inductor CD28 and CD80 which were downregulated at this time point, as were SLAMF-1 and BTLA. Due to the low number of study subjects and the missing follow-up period this topic should be further investigated, as should its clinical relevance during future long-term spaceflight missions (43).

Conclusion

This field study at *Concordia* investigated the degree and the dynamics of innate and adaptive immune changes in healthy men as a function of long-term isolation and confinement living under hypobaric hypoxia. The key observation is a two-step sensitization of different immunological components. The strongly enhanced immune responses when challenged with recall antigens and other receptor-dependent and -independent acting stimuli indicated a disinhibition of immune controlling functions of the innate and adaptive

branches. Unexpectedly, exposure to longterm hypoxia did not result in similar immunosuppressive consequences to acute hypoxia as a result of purinergic and HIF-1 α pathways.

Facing the next episode of human space exploration missions with future lunar/Martian habitats one should critically observe sensitization of the human immune system as a consequences of atmospheric conditions. This understanding can provide also a translational understanding on how these effects of long-time exposure of humans to hypoxia in such isolated environments may become relevant to understand also clinically relevant conditions and pathologies.

Limitations, strengths, and reliability of the findings

This study was selected after international competitive peer review and feasibility assessments by the polar institutes as well as ESA and established over two subsequent years at the *Concordia* station with access to the station only in the Antarctic summer (November until mid-February). The ESA sponsored research MDs were neither specialists in laboratory research nor experts in flow cytometry. Therefore, all preparations and weeklong trainings needed to be conducted in Europe and during the Antarctic summer. During the winter, the data verification and assay success was only possible by sending screen shots at the defined connection time points during the day. These conditions did not allow for adjustment of the protocol between the seasons, as supplies were shipped months ahead and resupply was not possible during the winter. The limitation of blood assays include blood volume restrictions, and allocated work time devoted this project by the research MD alongside the other ESA research protocols. As a result, it was only possible to work with whole blood. No complimentary assays with PBMCs or separations and single population

assays or analyses were feasible. This applies also to the fact that no population differentiation was possible for the transcriptomic assays. Given these limitations and the small sample sizes, all key assays were analyzed in a blinded fashion and observations were only considered relevant when they were observed in both seasons and when they reached a level of significance of p below an error rate of 5%. Furthermore, all other observed and presented findings were analyzed in three different independent laboratories (JSC, NASA; SCK•CEN, Belgium; University Hospital, LMU Munich, Germany) displaying the same direction/ cascade.

Disclosures

The authors have nothing to disclose. All authors declare that there is no conflict of interest.

Author contribution

M.F., B.C., G.S., M.T., S. B., C. Sa. and A.C. designed the work. M.F., B.C., A.S, A.R., A.C. collected the data. Data analysis and interpretation as well as drafting the article and critical revision for important intellectual content were performed by all authors (M. F., B.C., R.Q., J.-I. B., A.S., A.R., M.M., C.S., R.S., S.M., G.S., M.T., S.B., C.Sa., A.C.). All authors gave final approval of the version to be submitted and any revised version.

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References

1. Agency USCI. Antarctica - The World Factbook. 2018 17.01.2018 [cited 2018 27.02.2018]; Available from:
2. Feurecker M, Crucian B, Salam AP, Rybka A, Kaufmann I, Moreels M, et al. Early Adaption to the Antarctic Environment at Dome C: Consequences on Stress-Sensitive Innate Immune Functions. *High Alt Med Biol* 2014.
3. Crucian BE, Stowe RP, Pierson DL, Sams CF. Immune system dysregulation following short- vs long-duration spaceflight. *Aviat Space Environ Med* 2008;**79**(9):835-843.
4. de Jager W, Bourcier K, Rijkers GT, Prakken BJ, Seyfert-Margolis V. Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays. *BMC Immunol* 2009;**10**:52.
5. Kaufmann I, Hoelzl A, Schliephake F, Hummel T, Chouker A, Lysenko L, et al. Effects of adenosine on functions of polymorphonuclear leukocytes from patients with septic shock. *Shock* 2007;**27**(1):25-31.
6. Zhang Z. Missing data imputation: focusing on single imputation. *Ann Transl Med* 2016;**4**(1):9.
7. Tannheimer M, Fusch C, Boning D, Thomas A, Engelhardt M, Schmidt R. Changes of hematocrit and hemoglobin concentration in the cold Himalayan environment in dependence on total body fluid. *Sleep Breath* 2010;**14**(3):193-199.
8. Gunga HC, Kirsch KA, Roecker L, Kohlberg E, Tiedemann J, Steinach M, et al. Erythropoietin regulations in humans under different environmental and experimental conditions. *Respir Physiol Neurobiol* 2007;**158**(2-3):287-297.
9. Bradford A. The role of hypoxia and platelets in air travel-related venous thromboembolism. *Curr Pharm Des* 2007;**13**(26):2668-2672.
10. Lebedeva EV, Yushkov BG, Chereshev VA. Megakaryocytopoiesis under hypoxic conditions. *Bull Exp Biol Med* 2003;**136**(6):554-556.
11. Mei J, Liu Y, Dai N, Hoffmann C, Hudock KM, Zhang P, et al. Cxcr2 and Cxcl5 regulate the IL-17/G-CSF axis and neutrophil homeostasis in mice. *J Clin Invest* 2012;**122**(3):974-986.
12. Shirai T, Magara KK, Motohashi S, Yamashita M, Kimura M, Suwazomo Y, et al. TH1-biased immunity induced by exposure to Antarctic winter. *J Allergy Clin Immunol* 2003;**111**(6):1353-1360.
13. Shearer WT, Lee BN, Cron SG, Rosenblatt HM, Smith EO, Lugg DJ, et al. Suppression of human anti-inflammatory plasma cytokines IL-10 and IL-1RA with elevation of proinflammatory cytokine IFN-gamma during the isolation of the Antarctic winter. *J Allergy Clin Immunol* 2002;**109**(5):854-857.
14. Yadav AP, Mishra KP, Ganju L, Singh SB. Wintering in Antarctica: impact on immune response of Indian expeditioners. *Neuroimmunomodulation* 2012;**19**(6):327-333.
15. Rossol M, Heine H, Meusch U, Quandt D, Klein C, Sweet MJ, et al. LPS-induced cytokine production in human monocytes and macrophages. *Crit Rev Immunol* 2011;**31**(5):379-446.
16. Danis VA, Millington M, Hyland VJ, Grennan D. Cytokine production by normal human monocytes: inter-subject variation and relationship to an IL-1 receptor antagonist (IL-1Ra) gene polymorphism. *Clin Exp Immunol* 1995;**99**(2):303-310.

17. Volk AP, Barber BM, Goss KL, Ruff JG, Heise CK, Hook JS, et al. Priming of neutrophils and differentiated PLB-985 cells by pathophysiological concentrations of TNF-alpha is partially oxygen dependent. *J Innate Immun* 2011;**3**(3):298-314.
18. Drost EM, Kassabian G, Meiselman HJ, Gelmont D, Fisher TC. Increased rigidity and priming of polymorphonuclear leukocytes in sepsis. *Am J Respir Crit Care Med* 1999;**159**(6):1696-1702.
19. Chollet-Martin S, Montravers P, Gibert C, Elbim C, Desmonts JM, Fagon JY, et al. Subpopulation of hyperresponsive polymorphonuclear neutrophils in patients with adult respiratory distress syndrome. Role of cytokine production. *Am Rev Respir Dis* 1992;**146**(4):990-996.
20. Kaufmann I, Schachtner T, Feueracker M, Schelling G, Thiel M, Chouker A. Parabolic flight primes cytotoxic capabilities of polymorphonuclear leucocytes in humans. *Eur J Clin Invest* 2009;**39**(8):723-728.
21. Williams DL, Climie A, Muller HK, Lugg DJ. Cell-mediated immunity in healthy adults in Antarctica and the sub-Antarctic. *J Clin Lab Immunol* 1986;**20**(1):43-49.
22. Muller HK, Lugg DJ, Williams DL. Cutaneous immune responses in Antarctica. A reflection of immune status? *Arctic Med Res* 1988;**47 Suppl 1**:249-251.
23. Tingate TR, Lugg DJ, Muller HK, Stowe RP, Pierson DL. Antarctic isolation: immune and viral studies. *Immunol Cell Biol* 1997;**75**(3):275-283.
24. Eltzschig HK, Carmeliet P. Hypoxia and inflammation. *N Engl J Med* 2011;**364**(7):656-665.
25. Sitkovsky MV, Lukashev D, Apasov S, Kojima H, Koshiba M, Caldwell C, et al. Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A2A receptors. *Annu Rev Immunol* 2004;**22**:657-682.
26. Chouker A, Thiel M, Lukashev D, Ward JM, Kaufmann I, Apasov S, et al. Critical role of hypoxia and A2A adenosine receptors in liver tissue-protecting physiological anti-inflammatory pathway. *Mol Med* 2008;**14**(3-4):116-123.
27. Ohta A, Sitkovsky M. Extracellular adenosine-mediated modulation of regulatory T cells. *Front Immunol* 2014;**5**:304.
28. Blackburn MR. Too much of a good thing: adenosine overload in adenosine-deaminase-deficient mice. *Trends Pharmacol Sci* 2003;**24**(2):66-70.
29. Sitkovsky M, Lukashev D. Regulation of immune cells by local-tissue oxygen tension: HIF1 alpha and adenosine receptors. *Nat Rev Immunol* 2005;**5**(9):712-721.
30. Chen R, Liliental JE, Kowalski PE, Lu Q, Cohen SN. Regulation of transcription of hypoxia-inducible factor-1alpha (HIF-1alpha) by heat shock factors HSF2 and HSF4. *Oncogene* 2011;**30**(22):2570-2580.
31. Bigham AW. Genetics of human origin and evolution: high-altitude adaptations. *Curr Opin Genet Dev* 2016;**41**:8-13.
32. Bigham AW, Lee FS. Human high-altitude adaptation: forward genetics meets the HIF pathway. *Genes Dev* 2014;**28**(20):2189-2204.
33. Petousi N, Croft QP, Cavalleri GL, Cheng HY, Formenti F, Ishida K, et al. Tibetans living at sea level have a hyporesponsive hypoxia-inducible factor system and blunted physiological responses to hypoxia. *J Appl Physiol (1985)* 2014;**116**(7):893-904.
34. Goyal R, Longo LD. Acclimatization to long-term hypoxia: gene expression in ovine carotid arteries. *Physiol Genomics* 2014;**46**(19):725-734.
35. Baze MM, Schlauch K, Hayes JP. Gene expression of the liver in response to chronic hypoxia. *Physiol Genomics* 2010;**41**(3):275-288.

36. Detre C, Keszei M, Romero X, Tsokos GC, Terhorst C. SLAM family receptors and the SLAM-associated protein (SAP) modulate T cell functions. *Semin Immunopathol* 2010;**32**(2):157-171.
37. Menard L, Cantaert T, Chamberlain N, Tangye SG, Riminton S, Church JA, et al. Signaling lymphocytic activation molecule (SLAM)/SLAM-associated protein pathway regulates human B-cell tolerance. *J Allergy Clin Immunol* 2014;**133**(4):1149-1161.
38. Rethi B, Gogolak P, Szatmari I, Veres A, Erdos E, Nagy L, et al. SLAM/SLAM interactions inhibit CD40-induced production of inflammatory cytokines in monocyte-derived dendritic cells. *Blood* 2006;**107**(7):2821-2829.
39. Watanabe N, Gavrieli M, Sedy JR, Yang J, Fallarino F, Loftin SK, et al. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat Immunol* 2003;**4**(7):670-679.
40. Deppong C, Degnan JM, Murphy TL, Murphy KM, Green JM. B and T lymphocyte attenuator regulates T cell survival in the lung. *J Immunol* 2008;**181**(5):2973-2979.
41. Kobayashi Y, Iwata A, Suzuki K, Suto A, Kawashima S, Saito Y, et al. B and T lymphocyte attenuator inhibits LPS-induced endotoxic shock by suppressing Toll-like receptor 4 signaling in innate immune cells. *Proc Natl Acad Sci U S A* 2013;**110**(13):5121-5126.
42. Ye Z, Deng B, Wang C, Zhang D, Kijlstra A, Yang P. Decreased B and T lymphocyte attenuator in Behcet's disease may trigger abnormal Th17 and Th1 immune responses. *Sci Rep* 2016;**6**:20401.
43. Crucian B, Johnston S, Mehta S, Stowe R, Uchakin P, Quiarte H, et al. A case of persistent skin rash and rhinitis with immune system dysregulation onboard the International Space Station. *J Allergy Clin Immunol Pract* 2016;**4**(4):759-762 e758.

Tables:

	Baseline Europe	3-4 weeks	4 months	7 months	11 months
Hemoglobin [g/dL]	15.2 ± 1.0	17.1 ± 1.0*	18.4 ± 1.0*	17.8 ± 0.9*	17.5 ± 1.0*
Hematocrit [%]	46.4 ± 2.9	51.3 ± 3.4*	55.8 ± 4.9*	52.9 ± 3.5*	52.7 ± 3.6*
Thrombocytes [G/L]	216.7 ± 33.4	368.6 ± 75.2*	305.4 ± 72.9*	318.1 ± 92.2*	294.2 ± 44.7*
Leukocytes [G/L]	6.1 ± 1.4	7.1 ± 1.7	7.0 ± 1.3	7.0 ± 1.3	6.1 ± 1.7
Granulocytes [1000/μL]	3.1 ± 1.0	3.8 ± 1.2	3.8 ± 0.8*	3.7 ± 0.8*	2.9 ± 1.2
Granulocytes [%]	50.0 ± 6.9	53.3 ± 5.3	54.4 ± 6.9	52.1 ± 4.7	48.1 ± 8.9
Lymphocytes [1000/μL]	2.7 ± 0.9	3.3 ± 0.70*	3.2 ± 0.9*	3.3 ± 0.6*	3.2 ± 0.8*
Lymphocytes [%]	44.0 ± 8.8	46.7 ± 5.3	45.6 ± 6.9	47.9 ± 4.7	51.9 ± 8.9
Monocytes [1000/μL]	0.29 ± 0.09	0.33 ± 0.14	0.48 ± 0.21*	0.52 ± 0.25 (6)*	0.30 ± 0.13
Monocytes [%]	4.9 ± 1.6	4.8 ± 1.5	6.8 ± 2.2*	7.5 ± 3.1 (6)*	4.9 ± 1.9

Table 1 Complete/Differential blood cell count; Data are Mean values ± SD, (n), RM One way ANOVA followed by Holm-Sidak-test or ANOVA on ranks followed by Dunn's test, * p < 0.05 (tested vs. BDC).

	Baseline Europe	3-4 weeks	4 months	7 months	11 months
T Cells	66.8 ± 6.6	60.4 ± 7.0*	55.2 ± 8.9*	56.8 ± 6.8* (6)	55.7 ± 8.3*
B Cells	7.4 ± 6.6	13.1 ± 3.2*	11.5 ± 4.6	23.8 ± 16.9* (6)	13.4 ± 4.5*
NK Cells	6.2 ± 5.6	9.2 ± 8.2	12.2 ± 8.3	1.5 ± 1.8 (6)	10.9 ± 4.7
CD4+ T Cells	58.7 ± 8.5	54.7 ± 11.0*	51.5 ± 10.4*	58.2 ± 7.6 (6)	52.9 ± 6.5*
CD8+ T Cells	33.2 ± 7.2	31.6 ± 5.1	25.7 ± 4.6*	29.2 ± 3.3 (6)	29.8 ± 6.1
Bulk Memory CD4+	54.0 ± 13.0	58.6 ± 12.0*	59.1 ± 14.8	60.3 ± 13.7 (6)	68.3 ± 12.7
Bulk Memory CD8+	37.3 ± 9.6	59.0 ± 19.4*	58.0 ± 20.4*	38.8 ± 12.6 (6)	74.4 ± 7.6*
CD8: Naïve/ctx	85.0 ± 9.6	48.6 ± 20.7*	56.9 ± 15.2*	62.3 ± 26.0* (6)	52.9 ± 19.1*
CD8: Senescent	11.6 ± 9.8	35.2 ± 16.3*	26.4 ± 13.0*	29.8 ± 21.1* (6)	27.0 ± 16.3*
CD8: True Naïve	38.3 ± 17.2 (6)	34.7 ± 15.5	31.2 ± 8.1	42.7 ± 16.6 (6)	27.9 ± 7.9
Central Memory	6.0 ± 3.7 (6)	9.6 ± 3.6	13.3 ± 9.7*	7.2 ± 2.6 (6)	12.9 ± 5.8*
Effector Memory	38.8 ± 12.6 (6)	32.3 ± 14.2	33.2 ± 12.2	25.0 ± 12.4 (6)	34.7 ± 10.9
Term. Differentiated	17.2 ± 6.0 (6)	23.5 ± 7.2	22.5 ± 6.1	25.5 ± 6.0 (6)*	24.9 ± 5.0*
CD4/CD69	1.0 ± 0.7	6.0 ± 3.1*	1.7 ± 1.1	2.2 ± 2.1 (6)	2.0 ± 1.1
CD8/CD69	2.2 ± 1.1	9.1 ± 5.4*	2.7 ± 1.9	5.2 ± 2.2 (6)	2.8 ± 3.0
CD4/HLA-DR	2.0 ± 1.3 (6)	2.9 ± 2.6*	2.0 ± 1.3	1.4 ± 0.6 (5)	0.6 ± 1.0
CD8/HLA-DR	3.3 ± 2.5 (6)	4.4 ± 4.0*	1.5 ± 1.7	1.8 ± 1.1 (5)	0.7 ± 1.1

Table 2 Mean peripheral leukocyte distribution for *Concordia* overwinter subjects. Data are expressed as mean percentage ± SD. * indicates statistically significant difference p<0.05. RM One-way ANOVA followed by Holm-Sidak-test or ANOVA on ranks followed by Dunn's test.

Plasma cytokines	Baseline Europe	3-4 weeks	4 months	7 months	11 months
IL-1RA	1157.7 ± 792.1	965.0 ± 826.4	678.8 ± 528.0	642.9 ± 442.4	602.3 ± 320.6
G-CSF	2.6 ± 2.2	4.0 ± 0.9*	4.0 ± 1.3*	3.7 ± 1.2*	3.4 ± 1.4
CXCL/ENA-78	168.1 ± 132.8	522.7 ± 648.5	350.5 ± 300.4*	328.9 ± 245.0*	453.8 ± 495.9*
Spontaneous cytokine release 48h culture	Baseline Europe	3-4 weeks	4 months	7 months	11 months
TNF	2.7 ± 2.5	7.5 ± 9.4	93.6 ± 216.7*	107.9 ± 212.6*	68.3 ± 93.7*
IFN-γ	8.8 ± 12.1	4.0 ± 3.6	13.4 ± 22.6	15.9 ± 29.2	14.0 ± 18.7
IL-2	1.6 ± 4.7	1.5 ± 3.7	5.5 ± 9.7	7.0 ± 9.6	6.9 ± 9.6
IL-10	3.1 ± 3.1	4.3 ± 2.9	22.8 ± 56.1	14.5 ± 19.9	10.6 ± 14.3
LPS-Stimulation	Baseline Europe	3-4 weeks	4 months	7 months	11 months
TNF	158.2 ± 97.8	400.7 ± 317.3*	349.0 ± 301.9*	431.8 ± 380.7*	230.4 ± 165.6
IL-1β	1377.5 ± 516.0	2749.3 ± 1944.5	1455.2 ± 847.0	1550.4 ± 683.3	3594.6 ± 593.7*
IL-10	222.6 ± 62.8	470.8 ± 340.2*	668.7 ± 316.0*	656.3 ± 290.9*	103.7 ± 128.1

Table 3 Relevant changes of **plasma cytokine** concentrations (pg/mL) at the indicated time points during winterover at *Concordia* Station, RM One way ANOVA (Holm-Sidak method), further cytokines displayed in Supplemental Table 5.

Spontaneous cytokine release upon 48 hours native DMEM incubation (37°C), data are mean values ± SD, ANOVA on Ranks followed by Dunn's test.

LPS stimulation assay, Data are Mean concentrations [pg/mL] ± SD, RM One way ANOVA followed by Holm-Sidak-test, * p < 0.05 (tested vs. BDC).

Figures:

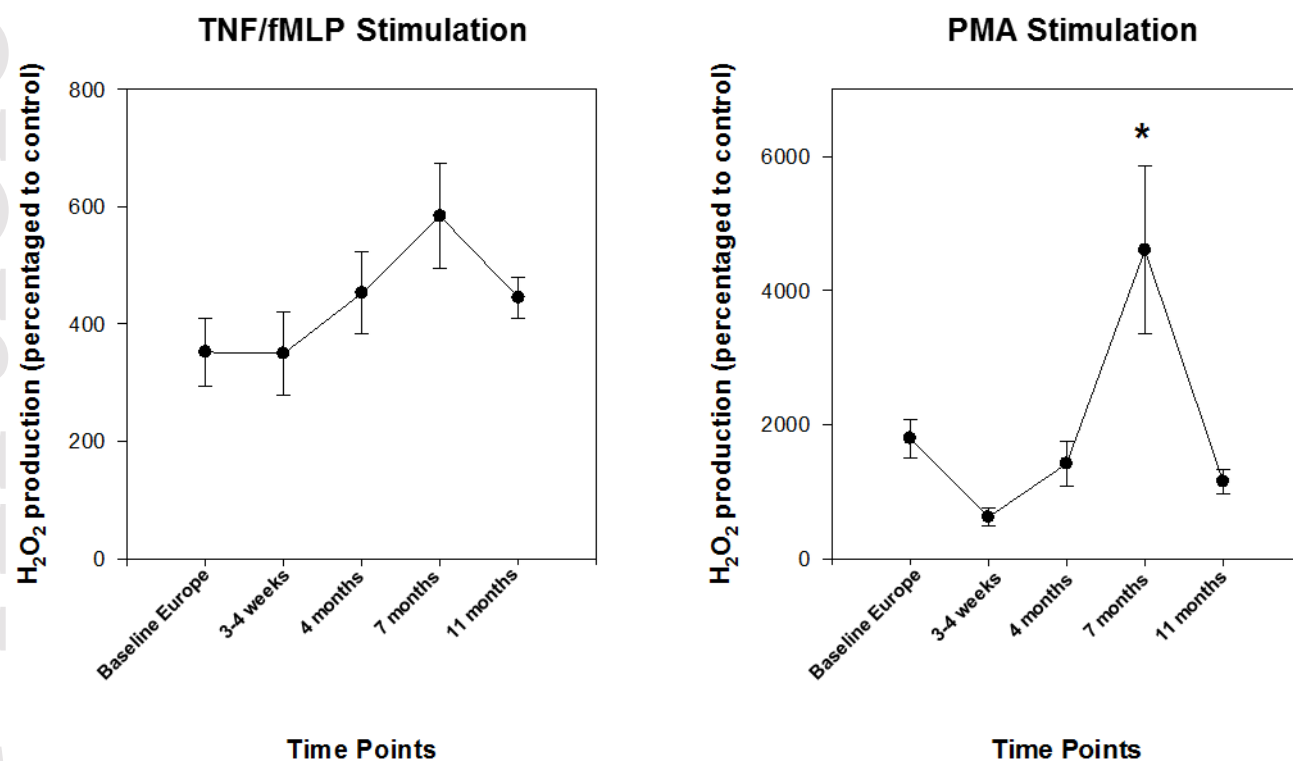
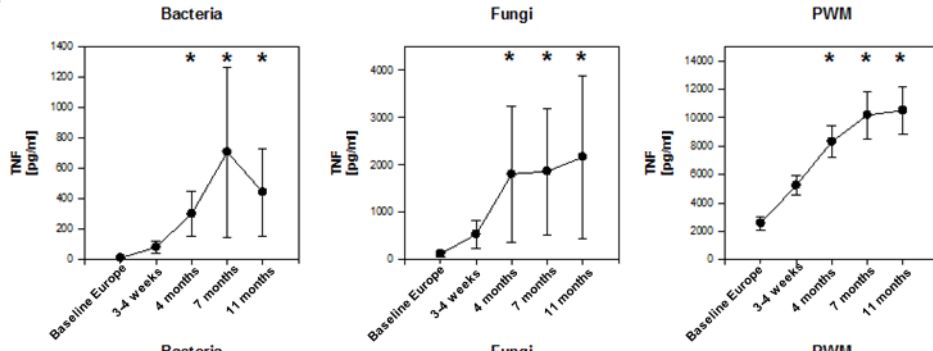
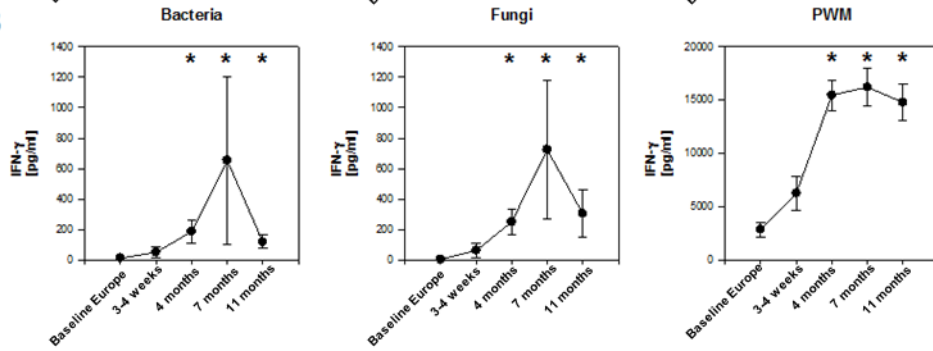


Figure 1 Stimulated granulocytotic (PMNL) oxidative burst; Data are mean values $\pm$ SEM, RM One way ANOVA followed by Holm-Sidak-test, * $p < 0.05$ (tested vs. BDC).

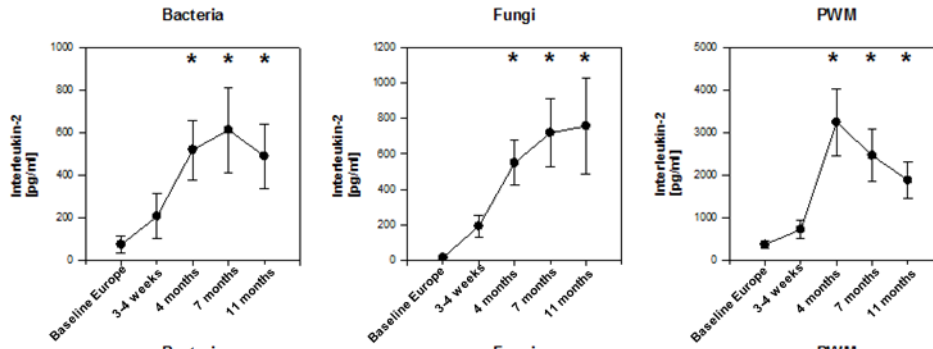
A



B



C



D

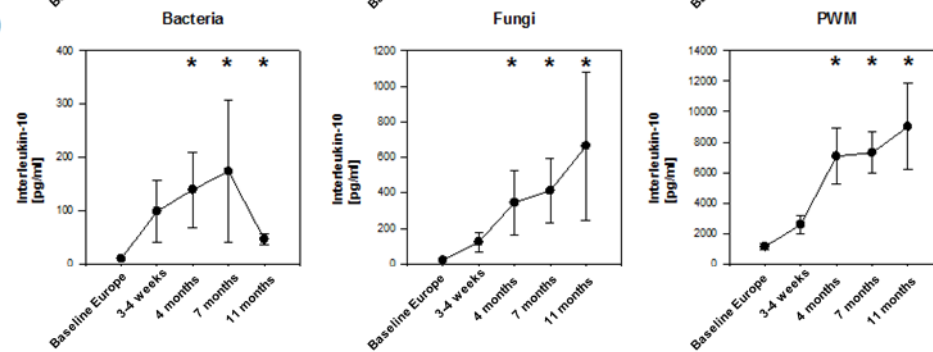
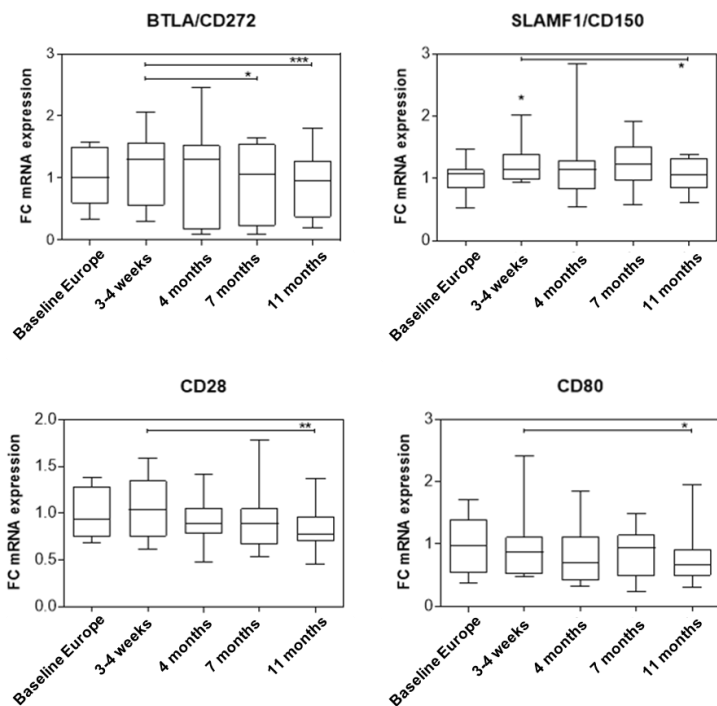


Figure 2 Re-call antigen and mitogen stimulation assay; **pro**-(A,B,C) and **anti**-(D)inflammatory cytokine profiles, data are mean values $\pm$ SEM, RM One way ANOVA followed by Holm-Sidak-test or ANOVA on ranks followed by Dunn's test, * $p < 0.05$ (tested vs. BDC).

A



B

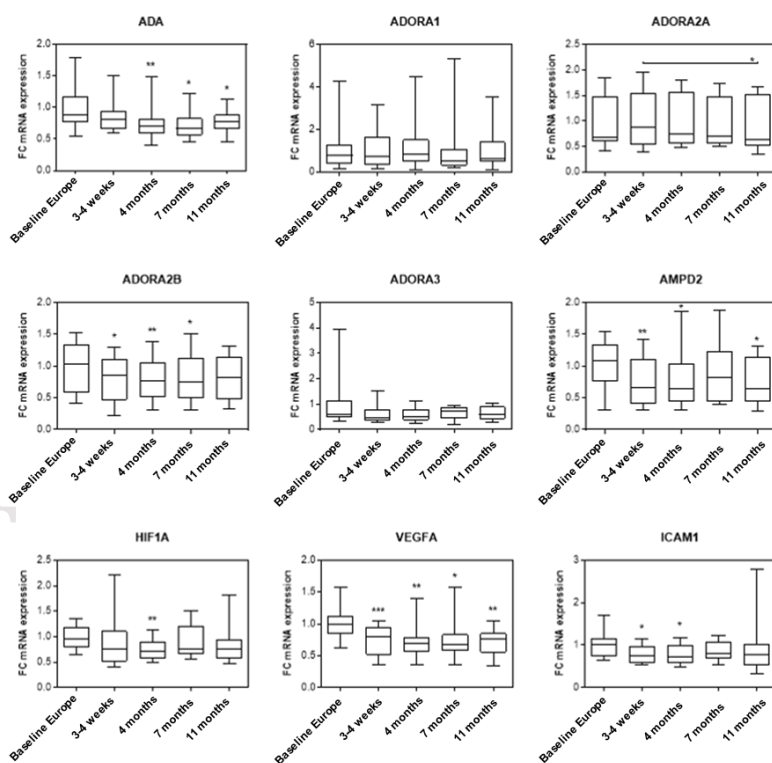
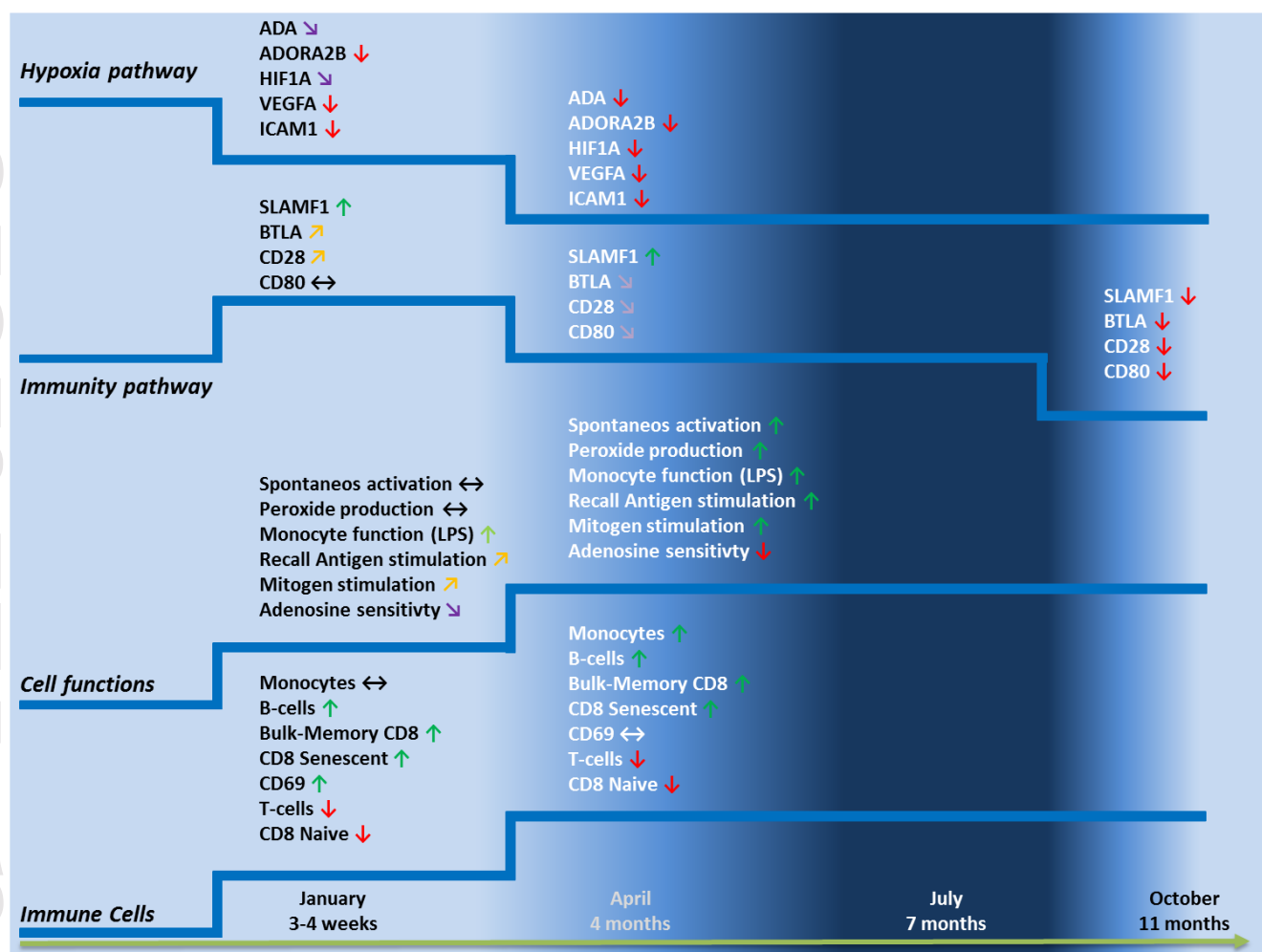


Figure 3 A) qRT-PCR for genes involved in immune pathways. Data are expressed as fold change (FC) normalized to expression levels of one subject at Baseline Europe.

B) qRT-PCR for genes involved in purinergic and hypoxia pathways. Data are expressed as fold change (FC) normalized to expression levels of one subject at Baseline Europe.

Centerlines show the median, boxes represent the range between first and third quartiles and whiskers represent the highest and lowest values. Asterisks represent significant differences (RM one-way ANOVA) compared to Baseline Europe unless otherwise indicated. * p < 0.05; ** p < 0.01; *** p < 0.001.



Graphical Abstract/ Figure 4

Overview of changes during one year at *Concordia* station.

Supplemental Material:

Methods

This study was approved by the ethics committee of the University of Munich [Reference Nr. 332-08] and Institutional Review Board for both the ESA and NASA elements of this scientific investigation. Informed written consent was signed by each participant prior to enrolment.

During the 2010 period, one previously enrolled subject resigned due to personal issues. One additional subject was enrolled immediately after deployment, in time to participate in the first deployed sampling. One participant was evacuated from Antarctica in February 2010 due to medical reasons. Hence, this subject was entirely excluded from this investigation. In total, 14 healthy male participants were analyzed for this investigation.

To exclude effects of hormonal changes females and because of the very low “n” of female overwintering crew (normally 1-2 per winter), female crew members were not enrolled for this study. Smokers were excluded to reduce the effects and bias of exogenous induced enzyme up-regulation.

Evaluation of immunocyte functional capacity/cytokine production following mitogenic stimulation

Concentrations of the prototypic T helper cell type 1 (Th1) cytokines IL-2, IFN- γ and TNF and the T helper cell type 2 (Th2) IL-10 were analyzed by Luminex xMAP® technology (Bioplex®) with commercially available reagents from BioRad Laboratories Inc. (Hercules, CA, USA) and Merck (Millipore) (Overijse, Belgium) according to the manufacturer's guidelines. Raw data processing was performed by using the Bioplex® software (Hercules, CA, USA) and xPONENT 3.1 Software (MV 's-Hertogenbosch, The Netherlands).

Expression of adhesion molecule CD11b upon fMLP stimulation

Additional testing for granulocyte functions were done using an ex vivo stimulation assay measuring the expression of CD11b. Whole blood was incubated for 15 min at 37°C before adding N-formyl-Methionyl-Leucyl-Phenylalanine (fMLP, 5×10^{-8} M). After additional 15 min incubation time at 37°C staining of CD11b (PerCP-Cy5.5 CD11b antibody, Becton Dickinson, Heidelberg, Germany) was performed. Antibody incubation occurred on ice water. After erythrocytal lysis flow cytometric measurement (Cyflow®, Partec, Münster, Germany) of CD11b on granulocytes was done. Identification of granulocytes occurred via the position in the forward/ side scatter (FSC/SSC) with subsequent quantification of CD11b expression in the forward versus FL3 scatter diagram.

Expression of CD11b was calculated as percent of spontaneous CD11b expression without any stimulation at each time point (see Supplemental Figure 2).

Confirming with the other ex vivo immune stimulation assays, stimulated CD11b expression peaked during deep winter (7months) before returning to almost baseline data. This complements the other data showing that immune functional properties were sensitized during the stay at *Concordia*.

Supplemental Tables:

	Winterover 2009	Winterover 2010	All/ both seasons
Number of participants	6	8	14
	<i>Mean ± SD</i>	<i>Mean ± SD</i>	<i>Mean ± SD</i>
	<i>(range)</i>	<i>(range)</i>	<i>(range)</i>
Age [years]	32.1 ± 9.2 (23.4 – 49.7)	38.3 ± 11.8 (24.9 – 57.4)	35.6 ± 10.8 (23.4 – 57.4)
Height [cm]	176.0 ± 5.4 (168 – 183)	179.0 ± 7.3 (172 – 193)	177.7 ± 6.5 (168 – 193)
Weight [kg]	72.8 ± 12.8 (62.4 – 98.0)	78.9 ± 10.8 (64.0 – 96.0)	76.3 ± 11.7 (62.4 – 98.0)
Body mass index [kg/m²]	23.6 ± 4.5 (19.0 – 32.0)	24.6 ± 3.0 (20.5 – 28.1)	24.2 ± 3.6 (19.0 – 32.0)

Supplemental Table 1 Demographic data of participants of the winterover campaigns 2009/2010, no significant differences between the groups in the two campaigns.

Time point		Month/year
Baseline Europe	Baseline data collection before deployment to Antarctica	October 2008 October 2009
3-4weeks	3-4 weeks at <i>Concordia</i> station	December/ January 2008/2009 December/ January 2009/2010
4 months	4 months at <i>Concordia</i> station	March/ April 2009 March/ April 2010
7 months	7 months at <i>Concordia</i>	June/ July 2009 June/ July 2010
11 months	11 months at <i>Concordia</i> station	October/ November 2009 October/ November 2010

Supplemental Table 2 Sample collection schedule for both seasons.

FITC	PE	PC5	Cell subsets identified
CD14	CD19	CD45	Leukocyte differential; B cells
CD3	CD56+16	CD45	Lymphocyte subsets; T, NK cells
CD4	CD8	CD3	T cell subsets; CD4:CD8
CD8	CD28	C1.7	CD8+ T cell subsets: active cytotoxic v. senescent
CD45RA	CD8	CD3	T cell subsets: Bulk memory:naïve
CD62L	CD45RA	CD8	T cell subsets: central memory
CD8	CD69	CD3	T cell subsets: constitutively activated (CD69+)
CD8	HLA-DR	CD3	T cell subsets: constitutively activated (HLA-DR+)
--	EBV/BMLF	CD8	Quantifies virus (EBV) peptide-specific CD8+ T cells
--	CMV/pp65	CD8	Quantifies virus (CMV) peptide-specific CD8+ T cells

Supplemental Table 3 Fluorochromes; Flow cytometry antibody matrix for Concordia Station.

Gene	Taqman Assay
18S	Hs99999901_s1
ADA	Hs01110949_m1
ADORA1	Hs00181231_m1
ADORA2A	Hs00169123_m1
ADORA2B	Hs00386497_m1
ADORA3	Hs00252933_m1
AMPD2	Hs01092053_g1
BTLA	Hs00699198_m1
CD28	Hs01007422_m1
CD80	Hs00175478_m1
HIF1A	Hs00153153_m1
RPLP0	Hs99999902_m1
SLAMF1	Hs00234150_m1
TBP	Hs00427620_m1
VEGFA	Hs00900054_m1

Supplemental Table 4 qPCR/ Taqman primer assays for each respective gene

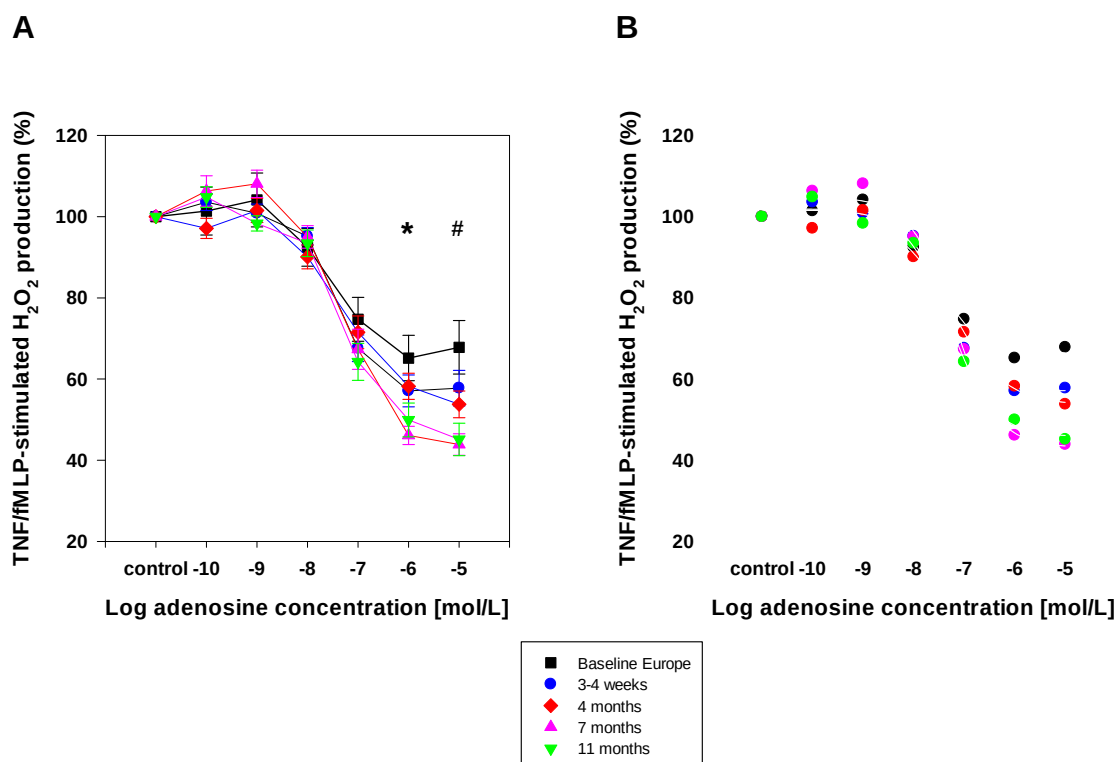
	Baseline Europe	3-4 weeks	4 months	7 months	11 months
IL-1a	0.1 ± 0.3	0.0 ± 0.2	0.1 ± 0.2	0.0 ± 0.1	0.0 ± 0.1
IL-1b	0.2 ± 0.4	0.3 ± 0.4	0.2 ± 0.6	0.2 ± 0.3	0.2 ± 0.3
TNF	1.2 ± 0.9	0.8 ± 0.8	0.9 ± 0.6	0.9 ± 0.8	0.7 ± 0.5
IL-6	0.1 ± 0.2	0.2 ± 0.2	0.3 ± 0.2	0.2 ± 0.3	0.2 ± 0.2
IL-8	7.6 ± 7.4	3.4 ± 2.0	3.0 ± 2.7	3.7 ± 3.3	5.0 ± 8.4
IFN-γ	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
IL-2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
IL-17	0.1 ± 0.2	0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
IL-4	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.6	0.7 ± 1.9	0.0 ± 0.0
IL-5	0.1 ± 0.1	0.1 ± 0.2	0.1 ± 0.1	0.0 ± 0.1	0.0 ± 0.0
IL-10	0.2 ± 0.1	0.3 ± 0.2	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.2
GM-CSF	0.1 ± 0.1	0.1 ± 0.2	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1
FGFb	0.0 ± 0.0	0.3 ± 1.1	1.9 ± 6.9	2.8 ± 4.6	2.3 ± 7.3
Tpo	322.8 ± 124.2	355.7 ± 131.9	338.0 ± 97.7	331.7 ± 219.7	343.1 ± 133.3
VEGF	9.8 ± 8.1	7.2 ± 4.9	6.7 ± 6.4	10.1 ± 13.7	8.6 ± 5.8
CCL2/MCP-1	108.6 ± 37.9	98.5 ± 61.5	93.0 ± 44.0	91.6 ± 60.5	90.7 ± 63.2
CCL3/MIP-1a	5.7 ± 14.0	4.3 ± 10.8	5.6 ± 11.7	6.8 ± 16.8	2.6 ± 9.4
CCL4/MIP-1b	24.1 ± 12.5	23.8 ± 13.1	21.6 ± 11.8	23.7 ± 15.1	25.2 ± 15.8
CCL5/RANTES	3356.3 ± 898.8	3256.6 ± 969.2	3068.4 ± 1003.0	3129.7 ± 1014.0	3099.4 ± 957.6

Supplemental Table 5 Plasma cytokine concentrations (pg/mL) at the indicated time points during winterover at *Concordia* Station, RM One way ANOVA (Holm-Sidak method).

Mitogen		Baseline Europe	3-4 weeks	4 months	7 months	11 months
SEA + SEB	CD4/69+	10 ± 1.2	21 ± 3.6	-	-	19 ± 2.4
	CD4/69/25+	2 ± 0.3	14 ± 3.8	-	-	7 ± 1.2
	CD8/69+	9 ± 1.4	25 ± 5.3	-	-	30 ± 3.9
	CD8/69/25+	2 ± 0.5	12 ± 4.1	-	-	7 ± 1.4
Anti- CD3/CD28	CD4/69+	48 ± 1.1	70 ± 0.6	-	-	52 ± 13.7
	CD4/69/25+	37 ± 1.2	49 ± 3.7	-	-	30 ± 11.9
	CD8/69+	51 ± 2.3	72 ± 5.9	-	-	42 ± 11.9
	CD8/69/25+	29 ± 2.7	40 ± 4.9	-	-	23 ± 11.1

Supplemental Table 6 Mean T cell function/early blastogenesis for Concordia overwinter subjects following mitogenic stimulation for 24 hours with the indicated mitogen. Data are expressed as mean percent cells stimulated to produce the indicated activation markers (CD69 and/or CD25) ± SD. For the Staphylococcal enterotoxins A/B (SEA/SEB) assay n=6, for the CD3/28 assay, n=4. RM One way ANOVA followed by Holm-Sidak-test, * p < 0.05 (tested vs. BDC).

Supplemental Figure:



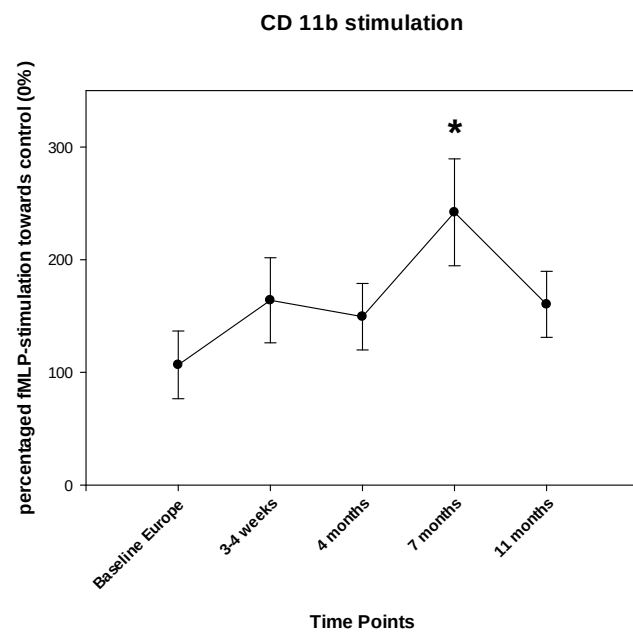
Adenosine	Baseline Europe	3-4 weeks	4months	7months	11months
IC 50 [nmol/L]	52.11	62.43	74.13	85.96	73.64

Supplemental Figure 1

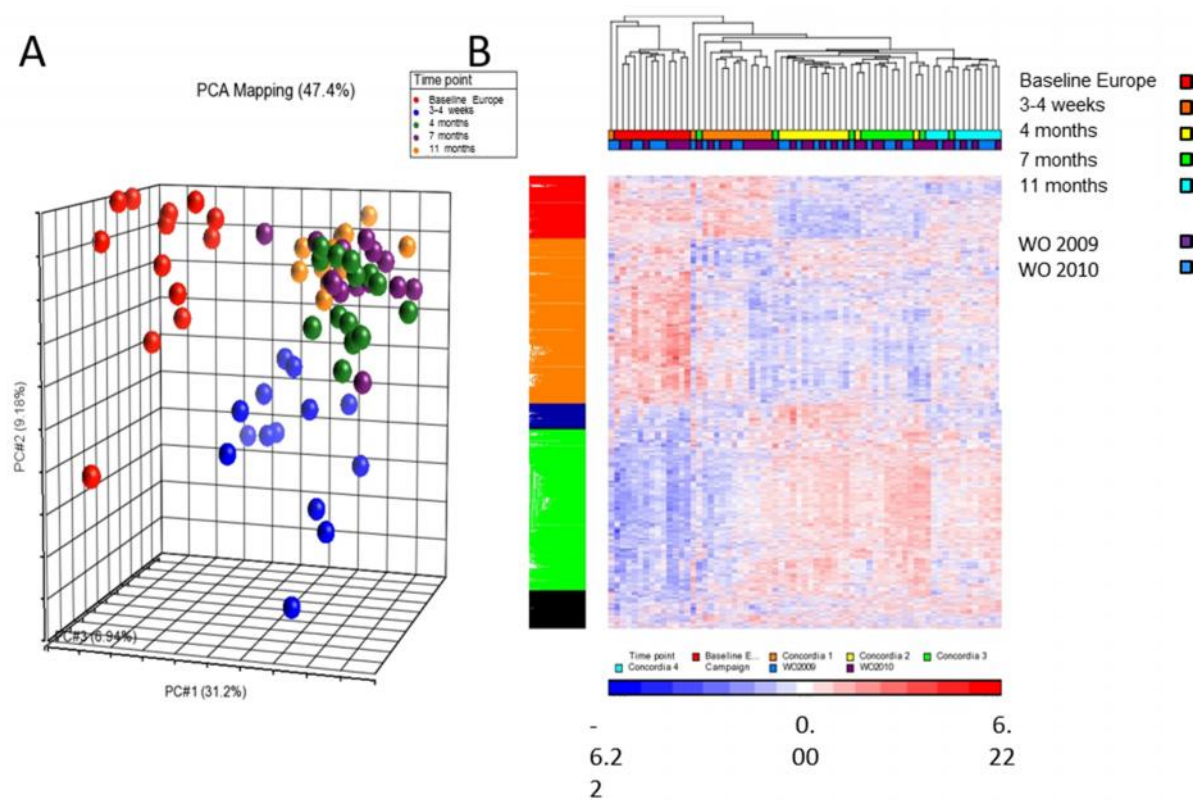
A) Measured dose-response curves for the inhibition of stimulated H_2O_2 production; data represent the mean percentage of $H_2O_2 \pm$ SEM; * $p < 0.05$ "Baseline Europe" vs. "7months" and "11months" (RM ANOVA on Ranks followed by Dunnetts' Method);

$p < 0.05$ BDC vs. 4, 7 and 11months (One way RM ANOVA followed by Holm-Sidak method);

B) Fitted dose-response curves for the inhibition of stimulated H_2O_2 production of polymorphonuclear leukocytes; Inhibitory concentration 50 (IC 50) values were assessed by logistic regression analyses of mean values of dose effects.



Supplemental Figure 2: fMLP stimulated CD11b expression on granulocyte as percent of unstimulated control: Data are mean $\pm$ SEM, * $p < 0.05$ (vs. BDC), BDC $n=7$, otherwise 13-14, One way RM ANOVA followed by Holm-Sidak method, * $p < 0.05$ (vs. BDC).



Supplemental Figure 3

Unsupervised hierarchical clustering and principal component analysis (PCA)