Impact of endotoxin exposure after exhausting exercise on the immune system in solid organ transplant recipients

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ABSTRACT

Subsequent to prolonged exhausting exercise a transient immunosuppression is often observed in athletes. This so-called "open window" results in a reduced resistance of the athletes to viral and bacterial infections after an exhaustive exercise bout. Concerning the effect of bacterial endotoxin contact after exhausting exercise in transplant recipients, who are innately immunosuppressed by their medication, no data exists at present. After performing 81 km cycling, including ascending more than 1800 m in altitude, peripheral blood from 10 male kidney transplant recipients and from 10 healthy controls matched for age and gender, was obtained. Simulating contact of the athletes with a pathogen post-exercise, the blood samples were incubated with Lipopolysaccharides (LPS). Thereafter, microarray analysis was performed. Microarray analysis revealed a markedly oppositional pattern of gene expression in transplant recipients compared with their controls after LPS incubation. Especially immune response genes were significantly over-represented in controls immediately after the exhaustive exercise bout with LPS stimulation, whereas numerous apoptotic genes were over-represented in transplant recipients. Merging our previous data with these recent findings it should be discussed if transplant recipients need to reduce their immunosuppressive medication before performing exhaustive exercise.

Key words: Exhaustive Exercise, Transplant recipients, Cycling, Immune system

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INTRODUCTION

It is well known that moderate exercise has a positive effect on the immune system, while exhausting exercise can lead to a transient immunodepression, a so-called "open window" resulting in a reduced resistance of the athlete to viral and bacterial infections after exhausting exercise (9; 11). Several conditions are suspected to be responsible to induce such an "open window" situation after an exhaustive exercise bout. For instance, white blood cells are activated and regulated following exhausting exercise. In regard to innate immunity, neutrophils are initially increased but after prolonged exercise a reduced degranulation and oxidative burst in response to bacterial stimulation has been observed (15). The number of circulating NK cells, which are important cells in first line of defence against infections, is also known to be reduced in blood following prolonged exercise (15). Concerning adaptive immunity, T helper (CD4+) type1 (Th1) cells play an important role in defence against intracellular pathogens (e.g. viruses) by cytokine release and stimulation of effector cells (15). Suzuki et al. were able to show that exhausting exercise can lead to the suppression of Th1 activity, by shifting the cytokine profile towards Th2 cells, inducing humoral immunity including allergic reactions (14). Furthermore, exhaustive exercise also leads to generation of reactive oxygen and nitrogen species (ROS, RNS) (10), with damaging potential and promotion of lymphocyte apoptosis (6).
All in all these conditions can lead to depression of immune function in athletes following exhaustive exercise. In the last edition of "Exercise Immunology Review" we had the opportunity to present data on the impact of exhaustive exercise on the immune response in transplant recipients (8). In regard to our previous findings and knowing about the "open window" after exhaustive exercise in healthy athletes it is of great interest for the transplant community, particularly since the immune system is strongly affected by the life-long required immunosuppressive medication after organ transplantation, to know what happens in transplant recipients after pathogen contact and exhausting exercise. In regard to simulating contact with a pathogen shortly after exhaustive exercise in transplant recipients, who are innately immunosuppressed by their medication, no data exists at present. We are delighted to present additional results from our previous study obtained during the „Euregio cycling tour 2009" in brief.

METHODS

As already described, the *"Euregio cycling tour"* is completed over 3 days and involves cycling more than 300 km (day 1: 110 km, day 2: 90 km, day 3: 102 km). Blood samples (2.5 ml whole blood) were drawn from seated subjects into PAXgene™ Blood RNA Tubes (Qiagen, Hilden, Germany) at rest before (t0) and immediately (up to 15 minutes) after cycling 81 km with an ascent of more than 1800 m in altitude from the starting point (t1). The whole blood samples in EDTA at (t1) were additionally incubated with Lipopolysaccharide (LPS) for 24 hours at room temperature. The final concentration of LPS used for stimulation of the whole blood samples was 1 ng·ml-1 (Sigma-Aldrich, St. Louis, USA). As already

mentioned the detailed candidate selection was described previously and is only summarized herein in brief. Out of approximately 30 solid organ transplant recipients, 10 male patients, all of whom had received a kidney transplant in their past were selected with a healthy control group, consisting of 10 cyclists, matched for age and gender. Only BMI showed a significant difference between groups (p=0.006, comtrols mean 78.0kg, transplant recipients mean 71.3kg) (8).
Immunosuppressive medication used daily by the transplant recipients included FK 506 (Tacrolimus), Cyclosporine, Mycophenolate-Mofetil or Azathioprine. Each subject gave written informed consent prior to participation in the study. The experimental protocols were approved by the Institute's Human Ethics Committee according to the principles set forth in the Declaration of Helsinki of the World Medical Association (449/2008BO2).

Transcriptome profiling

For expression profiling 400 ng of total RNA were linearly amplified and biotinylated using the Illumina® TotalPrep™ RNA Amplification Kits (Ambion) according to the manufacturer's instructions. Human HT-12v3 bead arrays (Illumina, San Diego, CA) were hybridized with 750ng cRNA for 18h at 58°C according to the Illumina® Whole-Genome Gene Expression with IntelliHyb Seal System Manual. Arrays were washed three times with buffer E1BC, High-Temp Wash Buffer and 100% ethanol, respectively, stained with streptavidine-Cy3 and again washed with buffer E1BC. Raw fluorescence intensities were recorded on a BeadArray Reader GX (Illumina). Average signal intensities, background correction, quantile normalization and quality control were performed with BeadStudio 3.1 software (Illumina).
All subsequent data analysis steps were performed on the software platform R 2.10.0 and Bioconductor 2.6.1 (5) with the packages "beadarray" (2; 4), "limma" (12; 13), "GOstats" (3). Initially, the expression data from all chips were normalized with VSN (7). The signal values were then averaged for the individual subgroups and differences in expression level were calculated. Differences between subgroups were extracted as contrasts and analyzed with the moderated F-test (empirical Bayes method) including a correction step for multiple testing with the 5%-FDR-based method of Benjamini and Hochberg. To attribute significant regulations to individual genes, a decision matrix was generated based on the function decide tests within the Limma option nestedF, where significant up- or down-regulations are represented by values of 1 or -1, respectively.
Due to the factorial design of the experiment, two parameters (patient group and time/treatment) have an impact on gene expression, while the influence of inter individual differences has to be taken into account. For both groups the factors time/treatment and donor was used to design a linear model capturing the influence of the different factors on gene expression levels. A non specific filter based on detection p-values was applied to remove non informative genes before the fitting of the linear models was performed. The coefficients describing the expression profiles of the remaining probe sets were calculated and the standard errors were moderated using an empirical bayesian approach. From the F statistic the resulting p-values were established and corrected for multiple testing with „Benjamini-Hochberg" (1).

Resulting gene lists were analyzed for over representation of gene ontology terms (www.geneontology.org) in the branches "biological process" and "molecular function" and KEGG pathways (www.kegg.jp) with conditional hypergeometric tests. Categories and pathways with a p-value less than 0.01 were considered significantly enriched. (8)

RESULTS

All cyclists, consisting of the 10 transplant recipients and the 10 corresponding healthy control athletes finished the tour safely and successfully. Both groups were comparable in their performance and none of the athletes fell ill during the tour. Simulating pathogen contact of the athletes post exercise, the blood samples were incubated with LPS. Thereafter, microarray analysis was performed. Microarray analysis revealed a markedly oppositional pattern of gene expression in transplant recipients compared with their controls after LPS incubation, directly after exhausting exercise (t1). Whereas 86 significantly up-regulated and 4 down-regulated genes were detected in transplant recipients (LPS treated vs untreated), we found an up-regulation of 151 and a downregulation of 18 significantly differentiated genes in the corresponding controls. Only 79 genes were regulated in common between these two groups.
Especially immune response genes were significantly over-represented in the controls immediately after the exhaustive exercise bout with LPS stimulation, whereas numerous apoptotic genes were over-represented in transplant recipients. Significantly over-represented gene ontology terms and KEGG pathways of the differentially expressed transcripts in transplant recipients and their corresponding controls are given in table 1.

DISCUSSION

Following prolonged exhaustive exercise a number of peripheral immunological parameters have been demonstrated to change significantly (16). These changes comprise induction of cytokines and hormones, ROS and RNS, changes in NK cell and in Th1 cell activity, leading so to a transient immunodepression, resulting in a reduced resistance of the athlete to viral and bacterial infections after exhausting exercise (9 – 11; 14; 16). Being aware of the fact, that this transient immunodepression ("open window") exists in healthy athletes after exhaustive exercise, the question has arisen in the transplant community as to what happens to transplant recipient athletes, who are innately immunosuppressed by their medication, after pathogen contact and exhausting exercise. We recently showed that the relative increase of neutrophils in transplant recipients was significantly smaller than in their corresponding controls after exhausting exercise and that concerning expression regulations the control athletes demonstrated a higher immune response regulation than the transplant recipients (8). Transplant recipients showed after exhaustive exercise a significant activation of genes related to cell metabolism, but genes related to the immune response were missing (8).
To further elucidate the answer to the question of what happens after pathogen

Table 1: Ontology terms significantly over-represented exclusively in transplant recipients and corresponding healthy control subjects, respectively, as well as collectively over-represented ontology terms in both groups, after the exhaustive exercise bout with LPS stimulation.

Over-represented ontologies in transplant recipients	**p-value**
regulation of apoptosis	< 0.001
regulation of cell death	< 0.001
negative regulation of apoptosis	< 0.001
regulation of cellular metabolic process	0.001
cell death	0.001
response to wounding	0.004
response to hyperoxia	0.004
induction of apoptosis	0.007
removal of superoxide radicals	0.008
recognition of apoptotic cell	0.008
cellular macromolecule biosynthetic process	0.009

Over-represented ontologies in corresponding healthy controls	**p-value**
toll-like-receptor 4 signaling pathway	< 0.001
negative regulation of Notch signalling pathway	< 0.001
lipopolysaccharide mediated signalling pathway	0.003
activation of innate immune response	0.004
negative regulation of transcription factor import into nucleus	0.005
regulation of NF-kappaB	0.006

Over-represented ontologies in both groups	**p-value**
cyclooxygenase pathway	0.001
adult somatic muscle development	0.001
oxidoreductase activity	0.002
prostaglandin-endoperoxide synthase activity	0.002
procollagen-proline 4-dioxygenase activity	0.004
prostaglandin E receptor activity	0.005

contact and exhausting exercise, we incubated peripheral blood samples from transplant recipients and their corresponding controls with LPS, followed by microarray analysis and are now delighted to present the results. Microarray analysis revealed a markedly oppositional pattern of gene expression in transplant recipients compared with their controls after LPS incubation, directly after exhausting exercise. Interestingly especially immune response genes were significantly over-represented in controls immediately after the exhaustive exercise bout with LPS stimulation, whereas numerous apoptotic genes were over-represented in transplant recipients. From our point of view this is a very disconcerting finding. We are aware of the fact that LPS incubation of peripheral blood is not exactly comparable to a real infection of the athlete, but nevertheless at least it gives a likely indication of how the immune system might respond to an infection. Con-

cerning the notable expression of apoptotic genes in transplant recipients, exhaustive exercise followed by pathogen contact, could lead to an increased risk of infection and cell damage in transplant recipients. Patients with long-life immunosuppressive medication are, however, known to be more prone to develop neoplasia over the course of time. Accordingly, additional apoptotic gene expression after exhaustive exercise may harbour an increased risk of cell failure with the potential consequence of promoting neoplasia. It seems possible that the immune system in transplant recipients is impaired by the effect of exhausting exercise, pathogen contact and additionally by the immunosuppressive medication. This could lead to an increased risk of infection in transplant recipients with potential cell damage and its consequences after exhaustive exercise. Whether a reduction of the immunosuppressive medication before exhaustive exercise could be advantageous remains unclear. From our point of view certain topics need to be urgently further elucidated. At first, investigations need to be done concerning the effect of moderate and regular exercise on the immune system in transplant recipients. It is possible that in transplant recipients, since inflammatory mechanisms are curbed by their medication, exercise has no pro-inflammatory effects and consequently no anti-inflammatory counter regulation and is therefore simply neutralized in its interaction with the immune system. On the other hand if regular moderate exercise shows an anti-inflammatory effect, could this be a possibility means of reducing the amount of immunosuppressive medication? Furthermore, the consequence of exhaustive exercise on its own and with pathogen contact after exhaustive exercise needs to be investigated in detail by immune function assays and expression and protein assays in transplant recipients.

Altogether our findings are the first to show these exciting gene expression alterations in transplant recipients after pathogen contact post-exhaustive exercise. Nevertheless our results raise new questions that are more than ever of great interest and impact for the transplant community particularly for those that do sports. Hence, this topic should be further investigated in the near future.

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REFERENCES

1. Benjamini Y and Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Proceedings of the Royal Statistical Society Series B 57: 289-300, 1995.
2. Dunning MJ, Smith ML, Ritchie ME and Tavare S. beadarray: R classes and methods for Illumina bead-based data. Bioinformatics 23: 2183-2184, 2007.
3. Falcon S and Gentleman R. Using GOstats to test gene lists for GO term association. Bioinformatics 23: 257-258, 2007.

4. Gautier L, Cope L, Bolstad BM and Irizarry RA. affy--analysis of Affymetrix GeneChip data at the probe level. Bioinformatics 20: 307-315, 2004.
5. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY and Zhang J. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 5: R80, 2004.
6. Hoffman-Goetz L and Quadrilatero J. Treadmill exercise in mice increases intestinal lymphocyte loss via apoptosis. Acta Physiol Scand 179: 289-297, 2003.
7. Huber W, von HA, Sultmann H, Poustka A and Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. Bioinformatics 18 Suppl 1: S96-104, 2002.
8. Königsrainer I, Zieker D, Löffler M, Bühler S, Walter M, Beckert S, Glatzle J, Northoff H, Nadalin S, Königsrainer A. Influence of exhaustive exercise on the immune system in solid organ transplant recipients. Exerc Immunol Rev. 2010;16:184-93.
9. Nieman DC and Nehlsen-Cannarella SL. The immune response to exercise. Semin Hematol 31: 166-179, 1994.
10. Niess AM, Dickhuth HH, Northoff H and Fehrenbach E. Free radicals and oxidative stress in exercise--immunological aspects. Exerc Immunol Rev 5: 22-56, 1999.
11. Radom-Aizik S, Leu SY, Cooper DM and Zaldivar F, Jr. Serum from exercising humans suppresses t-cell cytokine production. Cytokine 40: 75-81, 2007.
12. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 3: Article3, 2004.
13. Smyth GK. Limma: Linear Models for Microarray Data. In: Bioinformatics and Computational Biology Solutions using R and Bioconductor, edited by Gentelman R, Carey S, Dudoit S, Irizarry W and Huber W. New York: Springer, 2005, p. 397-420.
14. Suzuki K, Nakaji S, Kurakake S, Totsuka M, Sato K, Kuriyama T, Fujimoto H, Shibusawa K, Machida K, Sugawara K. Exhaustive exercise and type-1/type-2 cytokine balance with special focus on interleukin-12 p40/p70. Exerc Immunol Rev. 2003;9:48-57.
15. Walsh NP, Gleeson M, Shephard RJ, Gleeson M, Woods JA, Bishop NC, Fleshner M, Green C, Pedersen BK, Hoffman-Goetz L, Rogers CJ, Northoff H, Abbasi A, Simon P. Position statement. Part one: Immune function and exercise. Exerc Immunol Rev. 2011;17:6-63.
16. Zieker D, Fehrenbach E, Dietzsch J, Fliegner J, Waidmann M, Nieselt K, Gebicke-Haerter P, Spanagel R, Simon P, Niess AM and Northoff H. cDNA microarray analysis reveals novel candidate genes expressed in human peripheral blood following exhaustive exercise. Physiol Genomics 23: 287-294, 2005.