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Modulation of the peripheral immune system after low dose radon spa therapy: Detailed longitudinal immune monitoring of patients within the RAD-ON01 study

The pain relieving effects of low dose radon therapies on patients suffering from chronic painful inflammatory diseases have been described for centuries. Even though it has been suggested that low doses of radiation may attenuate chronic inflammation, the underlying mechanisms remain elusive. Thus, the RAD-ON01 study was initiated to examine the effects of radon spa therapy and its low doses of alpha radiation on the human immune system. In addition to an evaluation of pain parameters, blood was drawn from 100 patients suffering from chronic painful degenerative musculoskeletal diseases before as well as 6, 12, 18 and 30 weeks after the start of therapy. We verified significant long-term pain reduction for the majority of patients which was accompanied by modulations of the peripheral immune cells. Detailed immune monitoring was performed using a multicolor flow cytometry-based whole blood assay. After therapy, the major immune cells were only marginally affected. Nevertheless, a small but long-lasting increase in T cells and monocytes was observed. Moreover, neutrophils, eosinophils and, in particular, dendritic cells were temporarily modulated after therapy. Regarding the immune cell subsets, cytotoxic T and NK cells, in particular, were altered. However, the most prominent effects were identified in a strong reduction of the activation marker CD69 on T, B and NK cells. Simultaneously, the percentage of HLA-DR$^+$ T cells was elevated after therapy. The RAD-ON01 study showed for the first time a modulation of the peripheral immune cells following standard radon spa therapy. These modulations are in line with attenuation of inflammation.

Key words: radon spa, immune monitoring, chronic painful musculoskeletal diseases, attenuation of inflammation, immune system

Running title: Immune modulation by radon

Introduction

The beneficial effects of low-dose radiation therapy (LD-RT) using X-rays for patients suffering from chronic pain were already being described at the end of the 19th century (1). Reports of pain reduction after bathing in certain natural springs can be found from even earlier periods. In the early 20th century, the radioactive noble gas radon was found in many of these springs. Today, small doses of about 0.2–0.5 mSv of alpha radiation emitted by radon are believed to be responsible for analgesic and anti-inflammatory effects (2). Clinical improvements in inflammatory and degenerative diseases after exposure to low doses of radiation range from long-term pain reduction to complete analgesia (2-8). Nevertheless, the underlying mechanisms are still widely unknown (9).

An acute inflammatory response is a highly coordinated and protective process. It is accompanied by the five macroscopic signs pain, heat, swelling, redness, and loss of function, which reflect vasodilation and extravasation of immune cells into the target tissue. However, when this acute response fails to be resolved, chronic inflammation may persist. Normally, the extent of chronic inflammation is lower than during an acute response. Nevertheless, the affected patient suffers from the same macroscopic signs, whereby pain and loss of function may be the most prominent symptoms in painful degenerative diseases. Rheumatoid arthritis (RA) is also characterized by chronic inflammation, here of the synovium.

It is widely accepted that the immune system can be modulated by radiation (10, 11), and several pre-clinical observations prove that low doses of radiation attenuate existing inflammation or the inflammatory phenotype of immune cells (12-17). However, these investigations were mostly based on X-irradiation as this is prevalent in clinical applications, especially in the treatment of local chronic inflammatory diseases (18). In contrast, radon therapy is also applied for chronic multi-morbid disorders, but is only available in health spas with natural occurrences of this noble gas. Long-term effects on pain reduction have been observed for RA in particular (2). It has been proposed that

the immune system is involved in this radon-dependent pain reduction (19-21) and the Multidisciplinary European Low Dose Initiative (MELODI) has suggested interconnecting radiation research and immunology (22). However, detailed longitudinal analyses of the immune status of patients during radon spa therapy have not been conducted so far.

Consequently, the RAD-ON01 study presented here was initiated to explore for the first time the impact of low doses of alpha irradiation on the peripheral immune system during standard radon spa therapy. The peripheral blood of 100 patients with chronic painful degenerative musculoskeletal disorders was subjected to detailed immunophenotyping before and after radon spa therapy including follow-up for 7 months. For this purpose, we developed a modular assay for detailed immunophenotyping of peripheral human whole blood samples by multicolor flow cytometry (23). It allows the characterization of 34 different cell subsets covering all major immune cells such as T, B and NK cells, as well as dendritic cells (DCs), monocytes, neutrophils, eosinophils, basophils, and circulating hematopoietic stem cells. Furthermore, the activation status of these cells was evaluated.

Methods

Study design and patients

The RAD-ON01 study was a prospective and explorative trial with 103 patients suffering from chronic painful musculoskeletal disorders of the spine and/or joints (ethical approval: BLÄK #12131). Since two patients only attended the examination before therapy and one patient could not attend the final examination, all displayed data refer to the 100 patients for whom all immunophenotyping data was available. The patient characteristics are summarized in **Table 1**. All patients underwent radon spa therapy in March 2013 at the certified health resort Bad Steben (Bavaria, Germany) and were followed up for 30 weeks. A prerequisite for inclusion in the RAD-ON01 study was that all patients had a pain anamnesis of at least one year and had undergone previous drug treatments and/or

physiotherapy without lasting success. Only patients living in close proximity to Bad Steben were recruited to decrease the impact of environmental differences and to exclude placebo effects related to holiday benefits.

The study design was based on standard radon spa applications and in particular on former radon studies that had been conducted in Bad Steben (24). Thus, radon spa therapy was given as a series of nine baths (each 20 min, 34°C) in natural radon spring water (600 to 1,200 Bq/l) over 3 weeks (3 baths per week). The cumulative effective dose of radiation received in this radon spa treatment was estimated to be approximately 0.3 mSv (25). Complementary estimations of the radiation dose that reaches the tissue during radon spa therapy are currently being produced and examined in the GREWIS (genetic risks and the anti-inflammatory action of ionizing radiation) research project.

A placebo group could not be included in the RAD-ON01 study because of legal issues regarding radiation protection. Even though patients are allowed to undergo radon spa therapy after prescription, the situation is different from a legal perspective in a study including a placebo group where some patients would be sent into the radon spa. Other statutory provisions apply here. Nevertheless, we are currently working on setting up a RAD-ON02 study that will include a temporary placebo group (cross-over design).

Examination of the patients and follow-up

This radon spa therapy was applied as monotherapy. In order to estimate external influences, participants' residential situation, previous radon therapies and undesired but potential medication intake or other treatments were documented.

The patients were examined five times on site: before therapy as well as at 6, 12, 18, and 30 weeks after the start of therapy. They were examined for different pain and cardiovascular

parameters (not outlined here). The trigger point measurements by dolorimetry were only performed until week 18 due to fixed follow-up care agreements.

Measurement of pain parameters

The evaluation of individual pain perception was performed using visual analogue scales (VAS) ranging from 0 (no pain) to 10 (worst pain imaginable) that were filled out by every patient. To obtain additional objective pain parameters, dolorimetry was also performed. Therefore, eight pressure points were defined according to common practice and applicable pressure was measured to evaluate each patient's individual pain sensitivity (24). Moreover, all patients evaluated their individual pain development retrospectively at the end of the study using VAS.

Immunophenotyping: Data acquisition and analysis

Additionally, at all five time points, blood was drawn and transported to Universitätsklinikum Erlangen within 3 hours. All immunological investigations were performed here in the Laboratory of Radiation Immunobiology at the Department of Radiation Oncology. Upon arrival the blood was immediately processed for multicolor flow cytometric analyses by the established in-house DIoB assay (23).

Essentially, this DIoB assay comprises 11 staining panels requiring about 2.0 ml of whole blood. For each staining, 100 µl of blood were incubated with freshly prepared antibody mix. After incubation for 25 minutes in the dark at room temperature, erythrocytes were lysed and leukocytes were fixated in an automated 3-step process using the TQ-Prep Workstation (Beckman Coulter, Heidelberg, Germany). All samples were then washed twice with PBS and kept on ice in PBS containing 1% paraformaldehyde until measurement. For acquisition, the Gallios flow cytometer (Beckman Coulter) with 3 lasers in standard filter configuration was used. All 500 blood samples were processed by trained staff using previously established standard operating protocols, constant

cytometer settings and fresh antibody mixes.

The data obtained were analyzed using the Kaluza analysis software (v.1.2; Beckman Coulter). Since strong variations in blood sample conditions were detected at the fourth time point (18 weeks), most likely due to the high summer temperatures in July 2013, we excluded this time point from analysis in advance. The percentages of all cell subsets were then calculated in relation to total leukocytes or their respective major cell type (e.g. $CD4^+$ T helper cells out of all $CD3^+$ T cells) using MS Excel (Microsoft, Redmond, USA).

Statistical analysis

Statistical analyses were carried out using the IBM SPSS Statistics software (v.21.0.0.0, International Business Machines, Armonk, NY). For the immunophenotyping, the paired t test was used for statistical comparison of the data from time points 6, 12, and 30 weeks after treatment with the data from before therapy. For the pain parameters, all statistical analyses were also performed versus week 0 (before start of therapy) using the Friedman test followed by Wilcoxon correction for the VAS data and ANOVA or ANOVA with repeated measurements for the dolorimetry data of maximal and mean pressure points, respectively.

Results

Pain relief

The RAD-ON01 study demonstrated long-lasting pain reduction after radon spa therapy for the majority of patients (87%). Evaluation of the visual analogue scales (VAS), which were filled out by all patients at all time points, revealed a long-lasting and significant reduction of pain for the complete observation period (**Figure 1A**). Before therapy a mean VAS score of 5.1 was reached. This value decreased to a minimum of 4.2 (after 12 weeks) and remained at this lowered level for the rest of the observation period. Interestingly, at the end of the study this pain relief was evaluated as even

higher when the patients were asked to retrospectively estimate their pain progression (**Figure 1B**). They indicated a higher pain value before therapy (6.0 VAS points) than when asked on site. This pain reduction was experienced by 87% of all patients, whereby 18% had a transient and 69% a lasting effect (**Figure 1C**). This long-lasting pain reduction was confirmed by the more objective pressure point measurements (dolorimetry). An increase in applicable pressure representing a decrease in personal pain sensitivity was observed after radon spa therapy over the complete observation period (**Figure 1D**). This applied for the mean of all trigger points as well as for the one located in the most affected area. Furthermore, 81% of all patients stated that they would repeat this therapy and 96% said that they would recommend radon spa therapy to others.

Immune monitoring

Major immune cells

The composition of the major immune cells was hardly affected by the radon spa therapy (**Figure 2**). Nevertheless, a slight but long-lasting increase in T cells and monocytes was observed. The T cells increased on average from 22.5% to 24.2% (**Figure 2A**) and the monocytes from 6.2% to 6.9% (**Figure 2D**). Further on, neutrophils temporarily decreased slightly from an average of 55.6% to 53.2% shortly after therapy (**Figure 2G**). Simultaneously, eosinophils increased from 3.9% to 4.2% (**Figure 2H**).

Greater impact was determined on the DCs that circulate the periphery in very small numbers. They were directly determined as the plasmacytoid DC (pDC) and myeloid DC (mDC) subsets. The latter showed an increase of 21.2% (from 0.26% to 0.31% of all leukocytes, **Figure 2E**) whereas the number of pDC rose by 12.8% (from 0.15% to 0.17%; **Figure 2F**). Interestingly, a subdivision of mDCs into type I (mDC-1) and II (mDC-2) revealed that only the mDC-1 increased (of 21.8%, from 0.25% to 0.30%, not shown) and not the mDC-2. Collectively, the DCs temporarily

increased from 0.41% to 0.48%, which equals an increase of 18.2%. No effects were detected on B or NK cells (**Figure 2B-C**).

Immune cell subsets

The $CD4^+$ T helper cells (T_H; **Figure 3A**) and most of their subsets, including T_H1 (**Figure 3B**), T_H2 (**Figure 3C**) and T_H17 (not shown), did not show any alterations. The same was true for a functional distinction into naïve, effector, effector memory and central memory T_H (data not shown).

However, we identified a significant increase in T regulatory cells (T_{REG}) from 7.2% to 7.4% (in relation to T_H cells) shortly after therapy for up to 12 weeks (**Figure 3D**). This increase was more prominent when evaluated in relation to all cells as the number of T cells rose in general with a total increase of 12.7% (week 6: from 1.10% to 1.26%; not shown) to 23.6% (week 12: from 1.10% to 1.36%; not shown).

Considering the $CD8^+$ cytotoxic T cells (T_C), we found a small but significant decrease at later time points (**Figure 3E**) and shifts within the naïve, effector and memory subsets (**Figure 3F-I**). Collectively, a shift from the naïve and central memory T_C to the effector and effector memory T_C was identified. This shift paralleled the general decrease of T_C and started in week 12. The naïve T_C decreased by 7.8% from 24.4% to 22.5% (related to all T_C; **Figure 3F**) and the number of central memory T_C dropped strongly by 49.0% from 7.7% to 3.9% (**Figure 3H**). On the other hand, effector and effector memory cells gained about 8.6% (**Figure 3G**: from 40.4% to 43.9%) and 8.0% (**Figure 3I**: from 27.5% to 29.7%) respectively.

Furthermore, a shift within the three NK cell subsets which were determined by their CD56 and CD16 co-expression was revealed. A small but significant increase in the main cytotoxic NK subset ($CD56^{lo}/CD16^{hi}$; termed NK1) shortly after therapy (**Figure 3J**: from 91.3% to 92.5% related to all NK cells) was seen. Both other subsets decreased (**Figure 3K-L**). The smallest subset NK3

(CD56^{lo}/CD16^{-}) had a local minimum shortly after therapy (decrease from 2.7% to 1.8%), but recovered afterwards (**Figure 3L**). In contrast, NK2 (CD56^{hi}/CD16^{lo}) dropped continuously but very slightly throughout the observation period (**Figure 4K**: from 6.0% to 5.2%). These three subsets were also investigated for their co-expression of NK cell specific markers such as NKG2A (CD159a), NKG2C (CD159c), NKG2D (CD314) and CD94, but no modulations by radon spa therapy were found.

Moreover, no alterations within the B cell or monocyte subsets were observed. Likewise, we did not find any relationships between the therapy and the frequency of circulating hematopoietic stem cells (not shown).

Activation level of the immune cells

In addition to determining cell subset compositions, we were interested in the impact of radon spa therapy on the activation state of these circulating immune cells. Therefore, the expression of common activation markers such as CD38 (cyclic ADP ribose hydrolase), CD69 (very early activation antigen), CD80 (B7.1), CD86 (B7.2) and HLA-DR (MHC class II) was analyzed. The most prominent effects were found for CD69 and HLA-DR expression on lymphocytes (**Figure 4**). CD69 expression was strongly decreased on all lymphocytes with a local minimum between 6 and 12 weeks after the start of the radon spa therapy. Its expression level on T cells dropped by 34.0% (**Figure 4A**: from 15.7% to 10.3%, related to T cells), on B cells by 35.5% (**Figure 4B:** from 15.5% to 10.0%, related to B cells) and on NK cells by 45.8% (**Figure 4C**: from 29.1% to 15.8%, related to NK cells). In all three cases the expression of CD69 rose again at the end of the observation period, but was still lower than before therapy.

HLA-DR is a marker that is usually rare on T cells. Accordingly, with 2.7%, only a small proportion of T cells were expressing it. Nevertheless, this expression level continuously increased to an expression of 3.9% after six weeks and 4.3% after 30 weeks (**Figure 4D**). This equaled an increase of 32% to 58% and was a long-lasting effect. The investigation of this expression on the different T

cell subsets revealed variations between T_H and T_C. Shortly after therapy, HLA-DR expression on T_H rose from 2.2% to 2.9% (**Figure 4E**: increase of 29%) and even reached 3.1% (increase of 37%) at the end of observation period. Regarding T_C, the expression of HLA-DR was higher from the start (**Figure 4F**). Before therapy, 3.7% of all T_C were already expressing HLA-DR and this level rose even further to 5.9% after six weeks (increase of 59%) and 6.7% after 30 weeks (increase of 79%).

Discussion and conclusion

In the past, observations of long-lasting pain relief following radon spa therapy of patients with chronic painful degenerative diseases have been described (2, 3). This was also demonstrated in the multicenter IMuRa trial with approximately 680 patients. This study investigated the radon spa therapy commonly applied in various health spas in comparison with a control intervention for rheumatic outpatients and its results suggested beneficial analgesic effects of this therapy in rheumatic diseases for up to 9 months post-intervention (4). These observations were also confirmed by the explorative RAD-ON01 study with 100 patients presented here. Nevertheless, sufficient evidence of the beneficial effects of radon spa treatments has not yet been provided, mostly due to the poor quality of many of the studies performed (26).

Prior to execution of the RAD-ON01 study presented, we hypothesized that the low doses of alpha radiation emitted by radon might affect the immune system and thereby contribute to a reduction in or even resolution of chronic inflammation as the main cause of painful degenerative diseases. Since the blood functions as a means of transport for immune cells to reach their target tissue, one would expect that feasible immune modulation properties of radon could be detected not only in the inflamed tissues but also in the peripheral blood. We expected that these small doses of alpha radiation as applied in radon spa therapy induce small alterations to the immune status. Now, for the first time, the RAD-ON01 trial has demonstrated a modulation of the peripheral immune system through standard radon spa therapy (Figures 2-4). These modulations might contribute to the

pain relieving effects of the therapy, but conclusive proof of the underlying mechanisms still remains a challenge.

Neutrophils play a major role in inducing and maintaining inflammation. We found that the number of these innate immune cells temporarily decreased after radon spa therapy, which may indicate reduced tissue inflammation. Simultaneously, the number of eosinophils was elevated. Eosinophils are commonly viewed as non-specific destructive effector cells which play a major role in allergies and parasitic infections. However, it is now assumed that eosinophils have regulatory functions in tissue homeostasis and repair mechanisms (27, 28). One might speculate that an elevation of eosinophils could contribute to the restoration of tissue homeostasis in chronically inflamed tissues.

Furthermore, we found the DCs to be temporarily elevated. These highly efficient antigen-presenting cells circulate through the periphery constantly, capturing antigens and presenting them to T and B cells (29). The DCs express various pattern recognition receptors (PRRs) for classification of the captured antigens and, depending on this, their presentation is accompanied by either stimulatory or anti-inflammatory signals for T and B cells (29). Consequently, either adaptive immune responses or immune tolerance are induced, making DCs important regulators of the immune system. The blood DCs are differentiated into mDCs and pDCs, and both types were temporarily increased after the radon spa therapy. The mDCs express many different PRRs responding to several stimuli (29) while, in contrast, the pDCs are more specialized in sensing nuclear acids and fostering pro-inflammatory responses (30). A temporary increase in DCs, as observed in the RAD-ON01 study, may indicate a rise in the number of these effective regulators for active suppression or resolution of chronic inflammation and again for restoration of tissue homeostasis.

Regarding the main immune cells, we also found the number of T cells and monocytes to be slightly, but long-lastingly increased. This also applied to their expression of HLA-DR, which belongs

to the MHC class II complex and is generally expressed on professional antigen-presenting cells to show their captured antigens to T or B cells. Still, a few T cells express HLA-DR upon activation (31) and these cells have been found to capture and present autoantigens to other T cells and thus actively suppress them (32). In contrast to T cells, nearly all monocytes express HLA-DR, but an elevated expression level has been linked to a better therapy outcome in severe systemic inflammatory diseases (33, 34). Thus, an upregulation of HLA-DR on both cell types after radon exposure might actively contribute to resolving chronic inflammation.

We also detected modulations within the T cell subsets. Interestingly, no effects on the composition of T_H or its subsets were detected despite their central role in the coordination of innate and adaptive immune cells. Only the T_{REGs}, which are able to directly suppress the activation of other immune cells and are thus highly efficient and potent immune regulators even when occurring at very low frequency, were modulated. They play a significant role in the prevention of autoimmune diseases (35). Even tiny increases as observed here could evoke potent local or systemic immune suppression.

In contrast to T_H, we detected a late decrease in T_C and different shifts within its subsets from week 12 onwards. The main function of T_C is the cytotoxic destruction of infected or degenerated host cells. Therefore, the MHC class I complexes, which are expressed by all nucleated host cells and continuously present intracellular peptides, are screened for foreign (e.g. viral) or degenerated molecules (e.g. tumor proteins). Since these MHC class I molecules are expressed by nearly all host cells, defects in their recognition may cause extensive tissue damage. Indeed, a subgroup of $CD57^+$ T_C has already been shown to be elevated in patients with rheumatoid arthritis (36). However, the role of T_C in autoimmune diseases is still unclear and controversial (37), and animal studies on CD8 and CD4 deficient mice have suggested that T_C may instead have a regulatory or even protective function in arthritis (38). In the RAD-ON01 study presented here, we observed that T_C decreased in the

peripheral blood following the radon treatment, but further subtyping revealed shifts within the T_C subsets in the direction of effector subsets, suggesting an active contribution of T_C.

To summarize, we observed a late decrease of naive and central memory T_C in favor of effector and effector memory T_C. In general, the first two circulate through the body or reside in tissues waiting to encounter their specific antigen, but lack inflammatory and cytotoxic functions (39). Then upon challenge they proliferate extensively and generate effector or effector memory cells which then migrate into the target tissues for elimination of infected or degenerated cells accompanied by secretion of cytokines and chemokines (40). If available, the response of memory cells is much faster than that of naïve T cells (39) and it was observed that the effector memory cells increased earlier than the effector cells. However, occurring after around 12 weeks, all these effects appeared relatively late after radiation exposure, indicating that this response was a consequence of a previous occasion which was directly caused by radiation.

Lastly, shifts within the subsets of the innate NK cells were found even though their total number remained unaffected. The main cytotoxic NK1 subset (41) increased shortly after radon spa therapy and simultaneously the $CD56^{+}/CD16^{-}$ NK3 subset, which has not yet been functionally characterized, decreased. Unlike this early modulation, the $CD56^{hi}/CD16^{-}$ NK2 subset, which has a regulatory function and primarily supports other immune cells by cytokine secretion (e.g. IFNγ, TNFα, GM-CSF, IL-10, IL-13) (42), decreased continuously. However, we did not find any effects of the radon spa therapy on the expression of activating (NKG2c) or suppressing (NKG2a) molecules by NK cells (not shown). Thus, the role of NK cells remains elusive. However, this innate immune response might pave the way for the delayed T_C response.

In conclusion, the prospective and explorative RAD-ON01 study shows for the first time that immune modulations that may favor the attenuation of inflammation occur in the peripheral blood following radon spa therapy. One might speculate that radon and its secondary products might

contribute to the restoration of balance in chronically dysregulated inflammatory tissues. The latter are a major cause of clinical symptoms such as pain or joint stiffness. Certain immune modulations occurred only shortly after the end of therapy. However, some effects, in particular shifts within the T_C subsets, appeared later or remained for a long time, indicating secondary radiation effects. The deactivation of immune cells as observed in the reduced CD69 expression on T, B and NK cells may be responsible for the long-term improvement in clinical symptoms which was reported by the majority of patients.

A recently published randomized, placebo-controlled intervention study showed that exercise both with and without low-dose radon hyperthermia balneo treatment affected bone metabolism and quality of life in a study population of an age group at risk of developing osteoporosis (43). However, the patients in the therapy group had a slightly stronger reduction in the osteoclast-stimulating protein receptor activator of the nuclear κB-ligand (43). This study nicely illustrates the need for further randomized trials to investigate the effects of low doses of radon on the human body. As mentioned above, a RAD-ON02 study with a cross-over design is on the way. Taken together, the available studies expand the modes of action of radon to immune modulations and beneficial potential on bone metabolism. It is highly probable that osteo-immunological mechanisms (44) are influenced by radon spa therapy.

Location information

All authors are located in Germany. The entire study was conducted in Bad Steben, which is located in Bavaria in southern Germany. All blood investigations were performed in Erlangen, which is also located in Bavaria in southern Germany.

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Disclosure statement

The authors have nothing to declare.

References

1. Sokoloff, N. 1898. Röntgenstrahlen gegen Gelenkrheumatismus. *Wien Med Wochenschr* 12.
2. Franke, A., L. Reiner, H. G. Pratzel, T. Franke, and K. L. Resch. 2000. Long-term efficacy of radon spa therapy in rheumatoid arthritis--a randomized, sham-controlled study and follow-up. *Rheumatology (Oxford, England)* 39: 894-902.
3. Falkenbach, A., J. Kovacs, A. Franke, K. Jorgens, and K. Ammer. 2005. Radon therapy for the treatment of rheumatic diseases--review and meta-analysis of controlled clinical trials. *Rheumatology international* 25: 205-210.
4. Franke, A., and T. Franke. 2013. Long-term benefits of radon spa therapy in rheumatic diseases: results of the randomised, multi-centre IMuRa trial. *Rheumatology international* 33: 2839-2850.
5. Ott, O. J., C. Jeremias, U. S. Gaipl, B. Frey, M. Schmidt, and R. Fietkau. 2015. Radiotherapy for benign achillodynia : Long-term results of the Erlangen Dose Optimization Trial. *Strahlentherapie und Onkologie : Organ der Deutschen Rontgengesellschaft ... [et al]* 191: 979-984.
6. Ott, O. J., C. Jeremias, U. S. Gaipl, B. Frey, M. Schmidt, and R. Fietkau. 2014. Radiotherapy for benign calcaneodynia: long-term results of the Erlangen Dose Optimization (EDO) trial. *Strahlentherapie und Onkologie : Organ der Deutschen Rontgengesellschaft ... [et al]* 190: 671-675.
7. Ott, O. J., S. Hertel, U. S. Gaipl, B. Frey, M. Schmidt, and R. Fietkau. 2014. The Erlangen Dose Optimization Trial for radiotherapy of benign painful shoulder syndrome. Long-term results. *Strahlentherapie und Onkologie : Organ der Deutschen Rontgengesellschaft ... [et al]* 190: 394-398.
8. Ott, O. J., S. Hertel, U. S. Gaipl, B. Frey, M. Schmidt, and R. Fietkau. 2014. The Erlangen Dose Optimization trial for low-dose radiotherapy of benign painful elbow syndrome. Long-term results. *Strahlentherapie und Onkologie : Organ der Deutschen Rontgengesellschaft ... [et al]* 190: 293-297.
9. Trott, K. R., and F. Kamprad. 1999. Radiobiological mechanisms of anti-inflammatory radiotherapy. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology* 51: 197-203.

10. Frey, B., S. Hehlgans, F. Rodel, and U. S. Gaipl. 2015. Modulation of inflammation by low and high doses of ionizing radiation: Implications for benign and malign diseases. *Cancer letters* 368: 230-237.
11. Manda, K., A. Glasow, D. Paape, and G. Hildebrandt. 2012. Effects of ionizing radiation on the immune system with special emphasis on the interaction of dendritic and T cells. *Frontiers in oncology* 2: 102.
12. Wunderlich, R., A. Ernst, F. Rodel, R. Fietkau, O. Ott, K. Lauber, B. Frey, and U. S. Gaipl. 2015. Low and moderate doses of ionizing radiation up to 2 Gy modulate transmigration and chemotaxis of activated macrophages, provoke an anti-inflammatory cytokine milieu, but do not impact upon viability and phagocytic function. *Clinical and experimental immunology* 179: 50-61.
13. Frischholz, B., R. Wunderlich, P. F. Ruhle, C. Schorn, F. Rodel, L. Keilholz, R. Fietkau, U. S. Gaipl, and B. Frey. 2013. Reduced secretion of the inflammatory cytokine IL-1beta by stimulated peritoneal macrophages of radiosensitive Balb/c mice after exposure to 0.5 or 0.7 Gy of ionizing radiation. *Autoimmunity* 46: 323-328.
14. Large, M., S. Hehlgans, S. Reichert, U. S. Gaipl, C. Fournier, C. Rodel, C. Weiss, and F. Rodel. 2015. Study of the anti-inflammatory effects of low-dose radiation: The contribution of biphasic regulation of the antioxidative system in endothelial cells. *Strahlentherapie und Onkologie : Organ der Deutschen Rontgengesellschaft ... [et al]* 191: 742-749.
15. Rodel, F., L. Keilholz, M. Herrmann, R. Sauer, and G. Hildebrandt. 2007. Radiobiological mechanisms in inflammatory diseases of low-dose radiation therapy. *International journal of radiation biology* 83: 357-366.
16. Gaipl, U. S., S. Meister, B. Lodermann, F. Rodel, R. Fietkau, M. Herrmann, P. M. Kern, and B. Frey. 2009. Activation-induced cell death and total Akt content of granulocytes show a biphasic course after low-dose radiation. *Autoimmunity* 42: 340-342.
17. Kern, P. M., and L. Keilholz. 2009. Radio-immunological mechanisms of anti-inflammatory treatment: is there a way from the past into the future? *Autoimmunity* 42: 337-339.
18. Niewald, M., M. H. Seegenschmiedt, O. Micke, S. Graeber, R. Muecke, V. Schaefer, C. Scheid, J. Fleckenstein, N. Licht, and C. Ruebe. 2012. Randomized, multicenter trial on the effect of radiation therapy on plantar fasciitis (painful heel spur) comparing a standard dose with a very low dose: mature results after 12 months' follow-up. *International journal of radiation oncology, biology, physics* 84: e455-462.
19. Kataoka, T. 2013. Study of antioxidative effects and anti-inflammatory effects in mice due to low-dose X-irradiation or radon inhalation. *Journal of radiation research* 54: 587-596.
20. Zdrojewicz, Z., and J. J. Strzelczyk. 2006. Radon treatment controversy. *Dose-response : a publication of International Hormesis Society* 4: 106-118.
21. Yamaoka, K., F. Mitsunobu, K. Hanamoto, S. Mori, Y. Tanizaki, and K. Sugita. 2004. Study on biologic effects of radon and thermal therapy on osteoarthritis. *The journal of pain : official journal of the American Pain Society* 5: 20-25.
22. Salomaa, S., K. M. Prise, M. J. Atkinson, A. Wojcik, A. Auvinen, B. Grosche, L. Sabatier, J. R. Jourdain, E. Salminen, S. Baatout, U. Kulka, H. Rabus, E. Blanchardon, D. Averbeck, and W. Weiss. 2013. State of the art in research into the risk of low dose radiation exposure--findings of the fourth MELODI workshop. *Journal of radiological protection : official journal of the Society for Radiological Protection* 33: 589-603.
23. Ruhle, P. F., R. Fietkau, U. S. Gaipl, and B. Frey. 2016. Development of a Modular Assay for Detailed Immunophenotyping of Peripheral Human Whole Blood Samples by Multicolor Flow Cytometry. *International journal of molecular sciences* 17.
24. Pratzel, H. G., and P. Deetjen. 1997. *Radon in der Kurortmedizin*. ISMH Verlag, Geretsried.

25. Deetjen, P., A. Falkenbach, D. Harder, H. Jöckel, A. Kaul, and H. von Philipsborn. 2005. *Radon als Heilmittel, Therapeutische Wirksamkeit, biologischer Wirkungsmechanismus und vergleichende Risikobewertung.* Verlag Dr. Kovac, Hamburg.
26. Santos, I., P. Cantista, and C. Vasconcelos. 2015. Balneotherapy in rheumatoid arthritis-a systematic review. *International journal of biometeorology*.
27. Jacobsen, E. A., R. A. Helmers, J. J. Lee, and N. A. Lee. 2012. The expanding role(s) of eosinophils in health and disease. *Blood* 120: 3882-3890.
28. Furuta, G. T., F. D. Atkins, N. A. Lee, and J. J. Lee. 2014. Changing roles of eosinophils in health and disease. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology* 113: 3-8.
29. Zanoni, I., and F. Granucci. 2010. Regulation of antigen uptake, migration, and lifespan of dendritic cell by Toll-like receptors. *Journal of molecular medicine (Berlin, Germany)* 88: 873-880.
30. Reizis, B., A. Bunin, H. S. Ghosh, K. L. Lewis, and V. Sisirak. 2011. Plasmacytoid dendritic cells: recent progress and open questions. *Annual review of immunology* 29: 163-183.
31. Salgado, F. J., J. Lojo, C. M. Fernandez-Alonso, J. Vinuela, O. J. Cordero, and M. Nogueira. 2002. Interleukin-dependent modulation of HLA-DR expression on CD4and CD8 activated T cells. *Immunology and cell biology* 80: 138-147.
32. LaSalle, J. M., K. Ota, and D. A. Hafler. 1991. Presentation of autoantigen by human T cells. *Journal of immunology (Baltimore, Md. : 1950)* 147: 774-780.
33. Antoniades, C. G., P. A. Berry, E. T. Davies, M. Hussain, W. Bernal, D. Vergani, and J. Wendon. 2006. Reduced monocyte HLA-DR expression: a novel biomarker of disease severity and outcome in acetaminophen-induced acute liver failure. *Hepatology* 44: 34-43.
34. Cheadle, W. G., M. J. Hershman, S. R. Wellhausen, and H. C. Polk, Jr. 1991. HLA-DR antigen expression on peripheral blood monocytes correlates with surgical infection. *American journal of surgery* 161: 639-645.
35. Buckner, J. H. 2010. Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory T cells in human autoimmune diseases. *Nature reviews. Immunology* 10: 849-859.
36. Arai, K., S. Yamamura, S. Seki, T. Hanyu, H. E. Takahashi, and T. Abo. 1998. Increase of CD57+ T cells in knee joints and adjacent bone marrow of rheumatoid arthritis (RA) patients: implication for an anti-inflammatory role. *Clinical and experimental immunology* 111: 345-352.
37. Alzabin, S., and R. O. Williams. 2011. Effector T cells in rheumatoid arthritis: Lessons from animal models. *Febs Lett* 585: 3649-3659.
38. Taneja, V., N. Taneja, T. Paisansinsup, M. Behrens, M. Griffiths, H. Luthra, and C. S. David. 2002. CD4 and CD8 T cells in susceptibility/protection to collagen-induced arthritis in HLA-DQ8-transgenic mice: implications for rheumatoid arthritis. *Journal of immunology (Baltimore, Md. : 1950)* 168: 5867-5875.
39. Lanzavecchia, A., and F. Sallusto. 2005. Understanding the generation and function of memory T cell subsets. *Current opinion in immunology* 17: 326-332.
40. Mittrucker, H. W., A. Visekruna, and M. Huber. 2014. Heterogeneity in the Differentiation and Function of CD8(+) T Cells. *Arch Immunol Ther Ex* 62: 449-458.
41. Jacobs, R., G. Hintzen, A. Kemper, K. Beul, S. Kempf, G. Behrens, K. W. Sykora, and R. E. Schmidt. 2001. CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells. *European journal of immunology* 31: 3121-3127.
42. Cooper, M. A., T. A. Fehniger, S. C. Turner, K. S. Chen, B. A. Ghaheri, T. Ghayur, W. E. Carson, and M. A. Caligiuri. 2001. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood* 97: 3146-3151.
43. Winklmayr, M., C. Kluge, W. Winklmayr, H. Kuchenhoff, M. Steiner, M. Ritter, and A. Hartl. 2015. Radon balneotherapy and physical activity for osteoporosis prevention: a

randomized, placebo-controlled intervention study. *Radiation and environmental biophysics* 54: 123-136.

44. Jimi, E., and S. Ghosh. 2005. Role of nuclear factor-kappaB in the immune system and bone. *Immunological reviews* 208: 80-87.

Table 1. Patient characteristics.

Patient [number]	
In total	100
Sex [number]	
Male	40
Female	60
Age [years]	
Mean	60.2
Range	28 – 75
Diagnosis [number]	
Spine only	38
Joints only	32
Spine and joints	22
Fibromyalgia	8

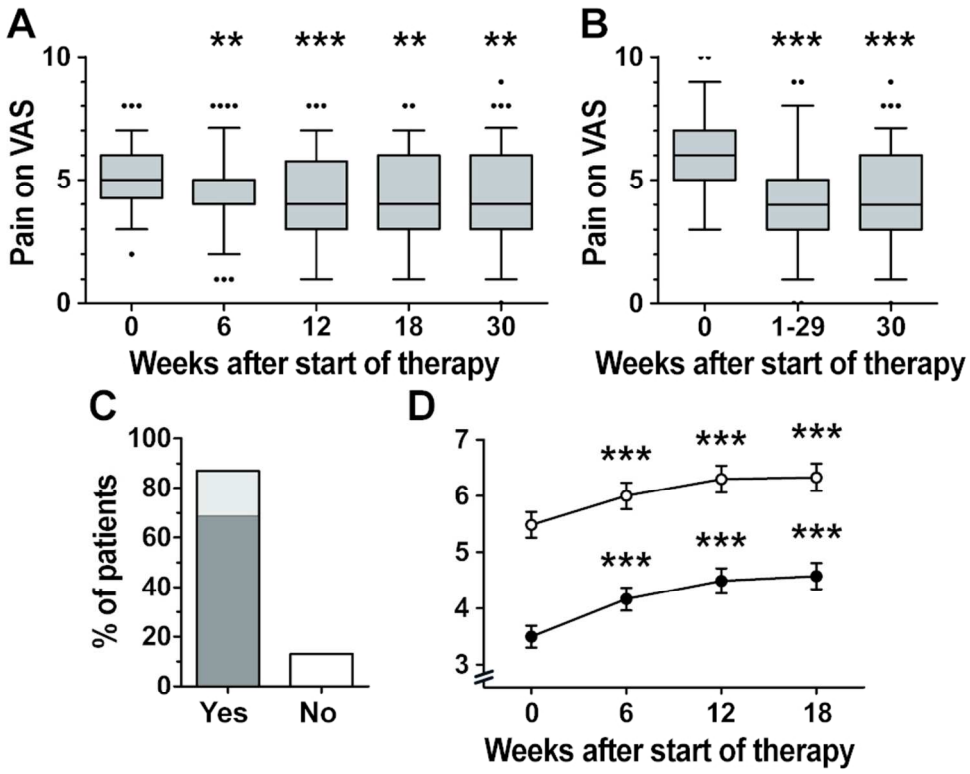
Legends to the figures

Figure 1: Long-lasting pain reduction following radon spa therapy. 100 patients determined their pain perception on a VAS ranging from 0 (no pain) to 10 (worst imaginable pain) on-site (**A**) as well as retrospectively in week 30 (**B**). 69% of patients had a long-lasting (**dark grey bar**) and 18% a sustained (**light grey bar**) effect (**C**). These subjective impressions were confirmed by pressure point measurements at previously defined trigger points (dolorimetry). The pressure required to induce pain increased steadily for both the mean of all 8 trigger points (**white circle**) and the one located in the most affected area (**black circle**) (**D**). Box and whisker plots (A, B), bar chart (C) or mean ± SEM (D). n = 100. All statistical analyses were performed vs. week 0 (before start of therapy) using the Friedman test with Wilcoxon correction (A,B), ANOVA (D: max. pressure point) or ANOVA with repeated measurements (D: mean pressure points). VAS: visual analogue scale; *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$.

Figure 2: Minor modulations of the major immune cells by radon spa therapy. The T cells (**A**) and monocytes (**D**) were slightly but long-lastingly increased after radon spa therapy. The B cells (**B**) and NK cells (**C**) were not affected. Regulatory DCs which were identified as mDCs (**E**) and pDCs (**F**) were temporarily increased shortly after therapy. Likewise, the neutrophils (**G**) and eosinophils (**H**) were affected shortly after therapy. Box and whisker plots. All statistical analyses were performed vs. time point 0 (before therapy) using the paired t test. *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$.

Figure 3: Modifications in several immune cell subsets by radon spa therapy. Many immune cell subsets were not affected by radon spa therapy, for example T_H (**A**) and most of its subsets such as T_H1 (**B**) or T_H2 (**C**); only T_{REGs} were affected and elevated for up to 12 weeks (**D**). In contrast to T_H, T_C was affected by the therapy and decreased from week 12 on (**E**). Moreover, T_C subsets revealed shifts from naïve (**F**) and central memory (**H**) subsets in the direction of the cytotoxic effector (**G**) and effector memory cells (**I**). Like the general T_C decrease, these shifts occurred relatively late after therapy. Furthermore, different shifts within the NK cell subsets were mainly identified shortly after therapy (**J–L**). Box and whisker plots. All statistical analyses were performed vs. time point 0 (before therapy) using the paired t test. *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$.

Figure 4: Major changes in the activation state of lymphocytes by radon spa therapy. The expression of the common activation CD69 was strongly reduced on T cells (**A**), B cells (**B**) and NK cells (**C**) after radon spa therapy. In contrast, HLA-DR expression was elevated on T cells (**D**), but differed between T_H (**E**) and T_C (**F**). Box and whisker plots. All statistical analyses were performed vs. time point 0 (before therapy) using the paired t test. *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$.



Long-lasting pain reduction following radon spa therapy. 100 patients determined their pain perception on a VAS ranging from 0 (no pain) to 10 (worst imaginable pain) on-site (A) as well as retrospectively in week 30 (B). 69% of patients had a long-lasting (dark grey bar) and 18% a sustained (light grey bar) effect (C). These subjective impressions were confirmed by pressure point measurements at previously defined trigger points (dolorimetry). The pressure required to induce pain increased steadily for both the mean of all 8 trigger points (white circle) and the one located in the most affected area (black circle) (D). Box and whisker plots (A, B), bar chart (C) or mean ± SEM (D). n = 100. All statistical analyses were performed vs. week 0 (before start of therapy) using the Friedman test with Wilcoxon correction (A,B), ANOVA (D: max. pressure point) or ANOVA with repeated measurements (D: mean pressure points). VAS: visual analogue scale; *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$.

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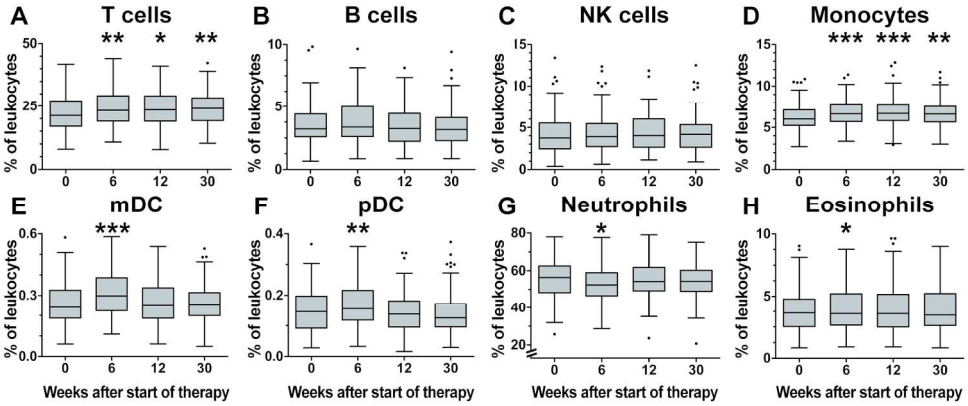

Minor modulations of the major immune cells by radon spa therapy. The T cells (A) and monocytes (D) were slightly but long-lastingly increased after radon spa therapy. The B cells (B) and NK cells (C) were not affected. Regulatory DCs which were identified as mDCs (E) and pDCs (F) were temporarily increased shortly after therapy. Likewise, the neutrophils (G) and eosinophils (H) were affected shortly after therapy. Box and whisker plots. All statistical analyses were performed vs. time point 0 (before therapy) using the paired t test. *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$.

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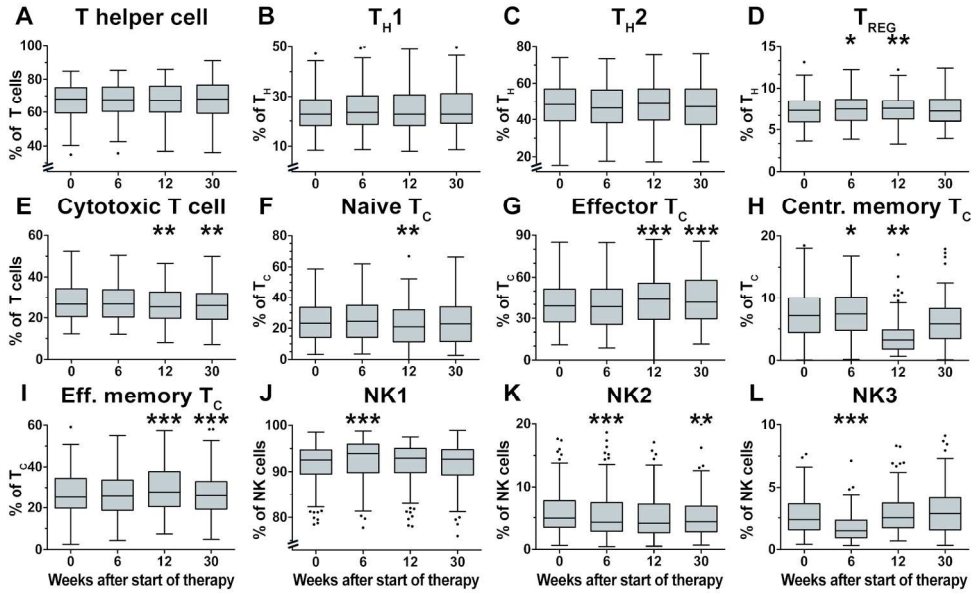

Modifications in several immune cell subsets by radon spa therapy. Many immune cell subsets were not affected by radon spa therapy, for example TH (A) and most of its subsets such as TH1 (B) or TH2 (C); only TREGs were affected and elevated for up to 12 weeks (D). In contrast to TH, TC was affected by the therapy and decreased from week 12 on (E). Moreover, TC subsets revealed shifts from naïve (F) and central memory (H) subsets in the direction of the cytotoxic effector (G) and effector memory cells (I). Like the general TC decrease, these shifts occurred relatively late after therapy. Furthermore, different shifts within the NK cell subsets were mainly identified shortly after therapy (J–L). Box and whisker plots. All statistical analyses were performed vs. time point 0 (before therapy) using the paired t test. *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$.

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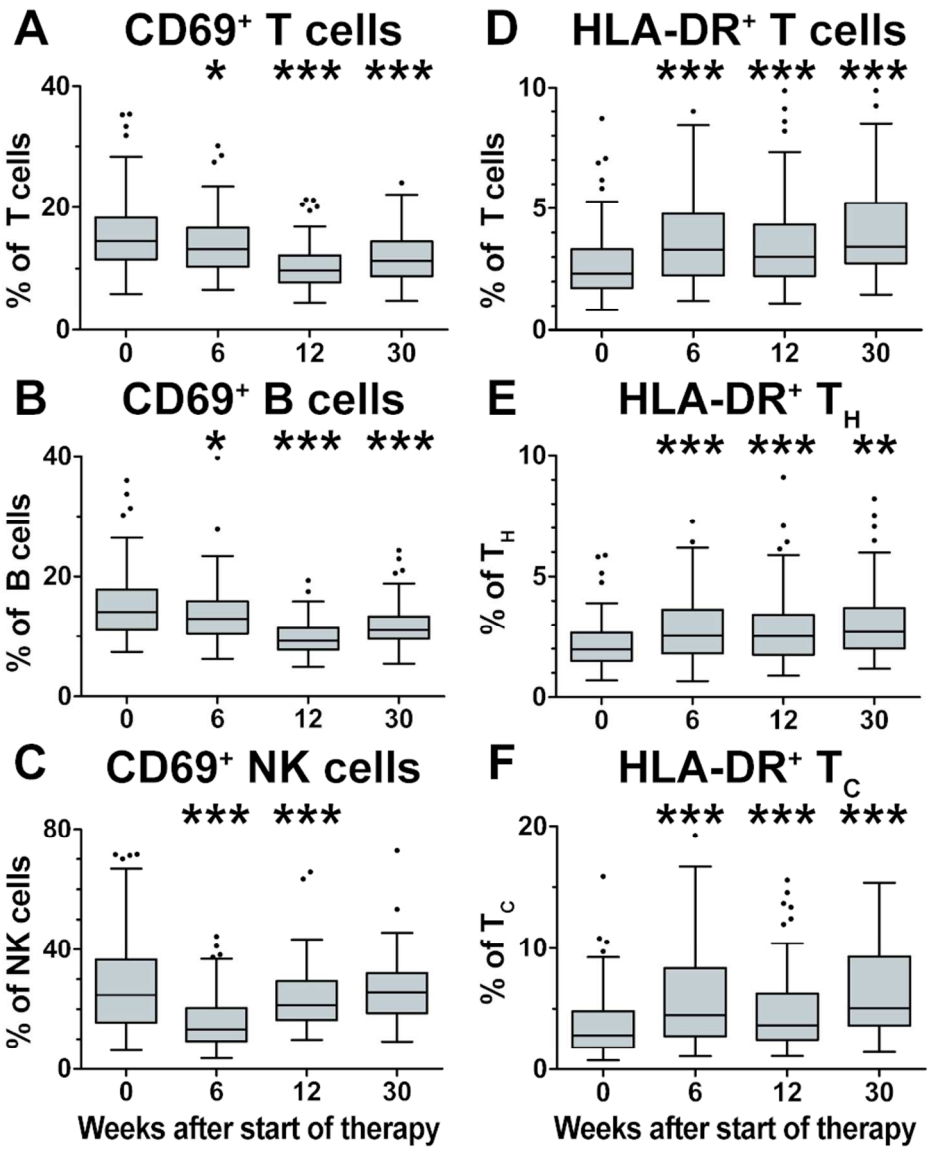

Major changes in the activation state of lymphocytes by radon spa therapy. The expression of the common activation CD69 was strongly reduced on T cells (A), B cells (B) and NK cells (C) after radon spa therapy. In contrast, HLA-DR expression was elevated on T cells (D), but differed between TH (E) and TC (F). Box and whisker plots. All statistical analyses were performed vs. time point 0 (before therapy) using the paired t test. *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$.

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Modulation of the peripheral immune system after low dose radon spa therapy: Detailed longitudinal immune monitoring of patients within the RAD-~~ON-01~~ON01 study

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Modulation of the peripheral immune system after low dose radon spa therapy: Detailed longitudinal immune monitoring of patients within the RAD-~~ON-01~~ON01 study

The pain relieving effects of low dose radon therapies on patients suffering from chronic painful inflammatory diseases have been described for centuries. Even though ~~an attenuation of chronic inflammations by~~it has been suggested that low doses of radiation ~~is suggested~~may attenuate chronic inflammation, the underlying mechanisms ~~of action still~~ remain elusive. Thus, the RAD-ON01 study was initiated to examine the effects of radon spa therapy and its low doses of alpha radiation on the human immune system. In addition to an evaluation of pain parameters, blood was drawn from 100 patients suffering from chronic painful degenerative musculoskeletal diseases before as well as 6, 12, 18 and 30 weeks after the start of therapy. We verified ~~a~~ significant long-term pain reduction for the majority of patients which was accompanied by ~~a modulation~~modulations of the peripheral immune cells. ~~The~~Detailed immune monitoring was performed using a multicolor flow~~-~~ cytometry~~-~~ based whole blood assay. After therapy, the major immune cells were only marginally affected. Nevertheless, a small but long-lasting increase ~~of~~in T cells and monocytes was observed. Moreover, neutrophils, eosinophils and ~~especially~~, in particular, dendritic cells were temporarily modulated after therapy. Regarding the immune ~~cells~~cell subsets, ~~especially~~ cytotoxic T and NK cells, in particular, were altered. ~~But~~However, the most prominent effects were identified in a strong reduction of the activation marker CD69 on T, B and NK cells. Simultaneously, the percentage of HLA-DR$^+$ T cells was elevated after therapy. The RAD-ON01 study showed for the first time a modulation of the peripheral immune cells following ~~a~~ standard radon spa therapy. These modulations are in line with ~~an~~ attenuation of inflammation.

Key words: radon spa, immune monitoring, chronic painful musculoskeletal diseases, attenuation of inflammation, immune system

Running title: Immune modulation by radon

Introduction

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The beneficial effects of low-dose radiation therapy (LD-RT) using X-rays for patients suffering from ~~chronical~~chronic pain were already being described ~~in~~at the end of the 19th century (1). ~~Even longer the~~Reports of pain reduction after bathing in certain natural ~~fountains was reported.~~springs can be found from even earlier periods. In the early 20th century, ~~in many of these fountains~~ the radioactive noble gas radon was found~~.~~ in many of these springs. Today, small doses of ~~its emitted~~ about 0.2–0.5 mSv of alpha radiation emitted by radon are believed to be responsible for analgesic and anti-inflammatory effects (2). Clinical improvements ~~of~~in inflammatory and degenerative diseases after exposure to low doses of radiation ~~are paralleled with~~range from long-term pain reduction ~~up~~ to complete analgesia (2-8). Nevertheless, the underlying mechanisms are still widely unknown (9).

An acute inflammatory response is a highly coordinated and protective process. It is accompanied by the five macroscopic signs pain, heat, swelling, redness, and loss of function, which reflect ~~the vasodilatation~~vasodilation and extravasation of immune cells into the target tissue. ~~But~~However, when this acute response fails to be resolved, ~~a~~ chronic inflammation ~~might~~may persist. Normally, the extent of ~~a~~ chronic inflammation is lower than during an acute response. ~~But still~~Nevertheless, the affected patient suffers from the same macroscopic signs, whereby pain and loss of function ~~might~~may be the most prominent symptoms in painful degenerative diseases. Rheumatoid arthritis (RA) is also characterized by chronic inflammation, here of the synovium.

It is widely accepted that the immune system can be modulated by radiation (10, 11), and several pre-clinical observations prove that low doses of radiation attenuate ~~an~~ existing inflammation or the inflammatory phenotype of immune cells (12-17). However, these investigations were mostly based on X-irradiation as this is prevalent in clinical applications, especially~~, for~~ in the ~~therapy~~treatment of local chronic inflammatory diseases (18). In contrast, radon therapy is also applied for chronic multi-morbid disorders, but is only available in health ~~resorts~~spas with natural

occurrences of this noble gas. Long-term effects on pain reduction have ~~particularly~~ been observed for RA in ~~RA~~particular (2). ~~An involvement of~~It has been proposed that the immune system ~~on~~is involved in this radon-dependent pain reduction ~~has been suggested~~ (19-21) and the Multidisciplinary European Low Dose Initiative (MELODI) has suggested interconnecting radiation research and immunology (22). ~~Unfortunately~~However, detailed longitudinal analyses of the immune status of patients during radon spa therapy have not been ~~missing~~conducted so far.

Consequently, the ~~here presented~~ RAD-ON01 study presented here was initiated to explore for the first time the impact of low doses of alpha irradiation on the peripheral immune system during ~~a~~ standard radon spa therapy. ~~Therefore, the~~The peripheral blood of 100 patients with chronic painful degenerative musculoskeletal disorders was subjected to detailed immunophenotyping before and after radon spa therapy including follow-up for 7 months. For this purpose, we ~~established~~developed a ~~multicolor flow cytometry-based~~modular assay~~, the~~ for detailed immunophenotyping of peripheral human whole blood ~~(DIoB) assay [Ruehle et al., Int J Mol Sci, under review], prior to this study.~~samples by multicolor flow cytometry (23). It allows the characterization of ~~40~~34 different cell subsets covering all major immune cells such as T, B and NK cells, as well as dendritic cells (DCs), monocytes, neutrophils ~~and~~, eosinophils ~~and~~, basophils, and ~~in addition~~ circulating hematopoietic stem cells. Furthermore, the activation status of these cells was evaluated.

Methods

Study design and patients

The RAD-ON01 study was a prospective and explorative trial with ~~100~~103 patients suffering from chronic painful musculoskeletal disorders of the spine and/or joints (ethical approval: BLÄK #12131). Since two patients only attended the examination before therapy and one patient could not attend

the final examination, all displayed data refer to the 100 patients for whom all immunophenotyping data was available. The patient characteristics are summarized in **Table 1**. All patients underwent ~~a~~ radon spa therapy in March 2013 ~~in~~at the certified health resort Bad Steben (Bavaria, Germany) and were followed up for 30 weeks. ~~Prerequisite~~A prerequisite for inclusion ~~into~~in the RAD-ON01 study was that all patients had a pain anamnesis of at least one year and ~~different medicinal and/or physiotherapeutic pre-~~had undergone previous drug treatments and/or physiotherapy without lasting success. Only patients living in close proximity to Bad Steben were recruited to decrease the impact of environmental differences and to exclude placebo effects related to holiday benefits.

The study design was based on standard radon spa applications and in particular on former radon studies that had been conducted in Bad Steben ~~(23).~~(24). Thus, radon spa therapy was given as a series of ~~9~~nine baths (~~à~~each 20 min, 34°C) in natural radon spring water (600 to 1~~.~~,200 Bq/l) over 3 weeks (3 baths per week). The cumulative effective dose of radiation received in this radon spa treatment was estimated to be approximately 0.3 mSv (25). Complementary estimations of the radiation dose that reaches the tissue during radon spa therapy are currently being produced and examined in the GREWIS (genetic risks and the anti-inflammatory action of ionizing radiation) research project.

A placebo group could not be included in the RAD-ON01 study because of legal issues regarding radiation protection. Even though patients are allowed to undergo radon spa therapy after prescription, the situation is different from a legal perspective in a study including a placebo group where some patients would be sent into the radon spa. Other statutory provisions apply here. Nevertheless, we are currently working on setting up a RAD-ON02 study that will include a temporary placebo group (cross-over design).

Examination of the patients and follow-up

This radon spa therapy was applied as monotherapy ~~and for estimation of~~ . In order to estimate external influences ~~the~~, participants' residential situation, previous radon therapies and undesired but potential medication intake or other treatments were documented.

The patients were examined five times on site: before therapy as well as at 6, 12, 18, and 30 weeks after the start of therapy. They were examined for different pain and cardiovascular parameters~~. Solely, the~~ (not outlined here). The trigger point measurements by dolorimetry were only performed until week 18 due to fixed follow-up care agreements. ~~Additionally, at all five time~~

Measurement of pain parameters

The evaluation of individual pain perception was performed using visual analogue scales (VAS) ranging from 0 (no pain) to 10 (worst pain imaginable) that were filled out by every patient. To obtain additional objective pain parameters, dolorimetry was also performed. Therefore, eight pressure points ~~blood was drawn and transported to the Universitätsklinikum Erlangen within 3 hours. Here, in the Laboratory of Radiation Immunobiology~~were defined according to common practice and applicable pressure was measured to evaluate each patient's individual pain sensitivity (24). Moreover, all patients evaluated their individual pain development retrospectively at the ~~Department of Radiation Oncology all immunological investigations were performed. Immediately upon arrival, the blood was processed for multicolor flow cytometric analyses by the in-house established DIoB assay.~~end of the study using VAS.

Immunophenotyping: Data acquisition and analysis

~~This~~Additionally, at all five time points, blood was drawn and transported to Universitätsklinikum Erlangen within 3 hours. All immunological investigations were performed here in the Laboratory of Radiation Immunobiology at the Department of Radiation Oncology. Upon arrival the blood was

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immediately processed for multicolor flow cytometric analyses by the established in-house DIoB assay (23).

Essentially, this DIoB assay comprises 11 staining panels requiring about 2.0 ml of whole blood. For each staining, 100 µl of ~~whole~~ blood were incubated with freshly prepared antibody mix ~~(see supplementary **Table S1**).~~. After incubation for 25 minutes in the dark at room temperature, erythrocytes were lysed and leukocytes were fixated in an automated 3-step~~-~~ process using the TQ-Prep~~-~~ Workstation (Beckman Coulter, Heidelberg, Germany). ~~Then, all~~All samples were then washed twice with PBS and kept on ice in PBS containing 1% paraformaldehyde until measurement. For acquisition, the Gallios flow cytometer (Beckman Coulter) with 3 lasers in standard filter configuration was used. All 500 blood samples were processed by trained staff using previously established standard operating protocols, constant cytometer settings and fresh antibody mixes. ~~An overview of the staining panels and the cytometer setting is provided in supplementary **Table S2** and supplementary **Table S3**, respectively.~~

The data obtained ~~data~~ were analyzed using the Kaluza analysis software (v.1.2; Beckman Coulter). Since strong variations ~~within~~in blood sample conditions were detected at the fourth time point (18 weeks), most likely due to the high summer temperatures in July 2013, ~~were detected, we in advance~~we excluded this time point from analysis~~. Then, the~~ in advance. The percentages of all cell subsets were then calculated in ~~dependence of~~relation to total leukocytes or ~~of~~ their respective major cell type (e.g. $CD4^+$ T helper cells out of all $CD3^+$ T cells) using MS Excel (Microsoft, Redmond, USA). ~~Finally, statistical~~

Statistical analysis

Statistical analyses were carried out using the IBM SPSS Statistics software (v.21.0.0.0, International Business Machines, Armonk, NY). ~~Here~~For the immunophenotyping, the paired t test was used for statistical comparison of the ~~remaining three~~data from time points ~~to its basic value~~6, 12, and 30

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weeks after treatment with the data from before therapy. For the pain parameters, all statistical analyses were also performed versus week 0 (before start of therapy) using the Friedman test followed by Wilcoxon correction for the VAS data and ANOVA or ANOVA with repeated measurements for the dolorimetry data of maximal and mean pressure points, respectively.

Results

Pain relief

The RAD-ON01 study demonstrated ~~a~~ long-lasting pain reduction after radon spa therapy for the majority of patients (87%). Evaluation of the visual analogue scales (VAS), which were filled out by ~~every patient~~all patients at all time points, revealed a long-lasting and significant reduction of pain for the complete observation period (**Figure 1A**). Before therapy a mean VAS score of 5.1 was reached. This value decreased to a minimum of 4.2 (after 12 weeks) and remained at this lowered level for the rest of the observation period. Interestingly, at the end of the study this pain relief was evaluated as even higher when the patients were asked to retrospectively estimate their pain progression (**Figure 1B**). They indicated ~~with 6.0 VAS points before therapy~~ a higher pain value ~~compared to~~before therapy (6.0 VAS points) than when asked on site. ~~Such a~~This pain reduction was experienced ~~for~~by 87% of all patients, whereby 18% had a transient and 69% a lasting effect (**Figure 1C**). This long-~~-~~lasting pain reduction was confirmed by the more objective pressure point measurements~~. Here, 8 trigger points were defined to common practice and applicable pressure was measured to evaluate the patient's pain sensitivity.~~ (dolorimetry). An increase in applicable pressure representing a decrease in personal pain sensitivity was observed after radon spa therapy over the complete observation period (**Figure 1D**). This applied for the mean of all trigger points as well as for ~~that~~the one located in the most affected area. Furthermore, 81% of all patients stated that they would repeat this therapy and ~~even~~ 96% said that they would recommend ~~the~~ radon spa therapy to others.

Immune monitoring

Major immune cells

The composition of the major immune cells was hardly affected by the radon spa therapy (**Figure 2**). Nevertheless, a slight but long-~~-~~lasting increase ~~of~~in T cells and monocytes was observed. The T cells increased ~~in~~on average from 22.5% to 24.2% (**Figure 2A**) and the monocytes from 6.2% to 6.9% (**Figure 2D**). Further on, neutrophils temporarily ~~slightly~~ decreased slightly from an average of 55.6% to 53.2% shortly after therapy (**Figure 2G**). Simultaneously, eosinophils increased from 3.9% to 4.2% (**Figure 2H**).

Greater impact was determined on the DCs that circulate the periphery in very small numbers. They were directly determined as the plasmacytoid DC (pDC) and myeloid DC (mDC) subsets. The latter showed an increase of 21.2% (from 0.26% to 0.31% of all leukocytes, **Figure 2E**) whereas the number of pDC rose ~~of~~by 12.8% (from 0.15% to 0.17%; **Figure 2F**). Interestingly, a subdivision of mDCs into type I (mDC-1) and II (mDC-2) revealed that only the mDC-1 increased (of 21.8%, from 0.25% to 0.30%, not shown~~), but~~) and not the mDC-2. Collectively, the DCs temporarily increased from 0.41% to 0.48~~%~~%, which equals an increase of 18.2%. No effects were detected on B or NK cells (**Figure 2B-C**).

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Immune cell subsets

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The $CD4^+$ T helper cells (T_H; **Figure 3A**) and most of ~~its~~their subsets, including T_H1 (**Figure 3B**), T_H2 (**Figure 3C**) ~~or~~and T_H17 (not shown), did not show any alterations. The same was true for a ~~functionally~~functional distinction into naïve, effector, effector memory and central memory T_H (data not shown).

However, we identified a significant increase ~~of~~in T regulatory cells (T_{REG}) from 7.2% to 7.4% (in relation to T_H cells) shortly after therapy for up to 12 weeks (**Figure 3D**). This increase was more

prominent when evaluated in relation to all cells as the number of T cells rose in general ~~and equaled~~with a total increase of 12.7% (week 6: from 1.10% to 1.26%; not shown) ~~up~~ to 23.6% (week 12: from 1.10% to 1.36%; not shown).

Considering the $CD8^+$ cytotoxic T cells (T_C), we found a small but significant decrease at later time points (**Figure 3E**) and shifts within the naïve, effector and memory ~~compartments~~subsets (**Figure 3F-I**). Collectively, a shift from the naïve and central memory T_C to the effector and effector memory T_C was identified. This shift paralleled the general decrease of ~~the~~ T_C and started in week 12. The naïve T_C decreased by 7.8% from 24.4% to 22.5% (related to all T_C; **Figure 3F**) and the number of central memory T_C dropped ~~even~~strongly by 49.0% from 7.7% to 3.9% (**Figure 3H**). On the other hand, effector and effector memory cells gained about 8.6% (**Figure 3G**: from 40.4% to 43.9%) and 8.0% (**Figure 3I**: from 27.5% to 29.7~~%),~~%) respectively.

Furthermore, a shift within the three NK cell subsets which were determined by their CD56 and CD16 co-expression was revealed. A small but significant increase ~~of~~in the main cytotoxic ~~main~~ NK subset ($CD56^{lo}/CD16^{hi}$; termed NK1) shortly after therapy (**Figure 3J**: from 91.3% to 92.5% related to all NK cells) was seen. Both other subsets ~~were decreasing~~decreased (**Figure 3K-L**). The smallest subset NK3 ($CD56^{lo}/CD16^{-}$) had a local minimum shortly after therapy (decrease from 2.7% to 1.8%), but recovered afterwards (**Figure 3L**). In contrast, ~~the~~ NK2 ($CD56^{hi}/CD16^{lo}$) dropped continuously but very slightly ~~during~~throughout the ~~complete~~ observation period (**Figure 4K**: from 6.0% to 5.2%). These three subsets were also investigated for their co-expression of NK cell specific markers such as NKG2A (CD159a), NKG2C (CD159c), NKG2D (CD314) and CD94, but no modulations by radon spa therapy were found.

Moreover, no alterations within the B cell or monocyte subsets were observed. Likewise, we did not find any ~~relations~~relationships between the therapy and the frequency of circulating hematopoietic stem cells (not shown).

Activation level of the immune cells

In addition to ~~the determination of~~determining cell subset compositions, we were ~~very~~ interested in the impact of radon spa therapy on the activation state of these circulating immune cells. Therefore, the expression of common activation markers such as CD38 (cyclic ADP ribose hydrolase), CD69 (very early activation antigen), CD80 (B7.1), CD86 (B7.2) and HLA-DR (MHC class II) ~~were~~was analyzed. The most prominent effects were found for ~~the~~ CD69 and HLA-DR expression on lymphocytes (**Figure 4**). ~~The~~ CD69 expression was strongly decreased on all lymphocytes with a local minimum between 6 and 12 weeks after the start of the radon spa therapy. Its expression level on T cells dropped by 34.0% (**Figure 4A**: from 15.7% to 10.3%, related to T cells), on B cells by 35.5% (**Figure 4B:** from 15.5% to 10.0%, related to B cells) and on NK cells by ~~even~~ 45.8% (**Figure 4C**: from 29.1% to 15.8%, related to NK cells). In all three cases the expression of CD69 rose again at the end of the observation period, but was still lower than before therapy.

~~The~~ HLA-DR is a marker ~~commonly~~that is usually rare on T cells. Accordingly, with 2.7~~%~~%, only a small ~~portion~~proportion of T cells were expressing it. Nevertheless, this expression level continuously increased to an expression of 3.9% after six weeks and ~~even~~ 4.3% after 30 weeks (**Figure 4D**). This equaled an ~~elevation~~increase of 32% ~~up~~ to 58% and was a long-lasting effect. The investigation of this expression on the different T cell subsets revealed variations between T_H and T_C. Shortly after therapy, ~~the~~ HLA-DR expression on T_H rose from 2.2% to 2.9% (**Figure 4E**: ~~elevation~~increase of 29%) and even reached 3.1% (increase of 37%) at the end of observation period. Regarding ~~the~~ T_C, the expression of HLA-DR was higher from ~~begin on~~the start (**Figure 4F**). Before therapy, ~~already~~ 3.7% of all T_C were already expressing HLA-DR and this level rose even further ~~up~~ to 5.9% after six weeks (increase of 59%) and 6.7% after 30 weeks (increase of 79%).

Discussion and conclusion

In the past, observations of ~~a~~ long~~-~~lasting pain relief following radon spa therapy of patients with

chronic painful degenerative diseases ~~were repeatedly~~have been described (2, 3). This was also ~~shown by~~ demonstrated in the multicenter IMuRa trial with ~~about~~approximately 680 patients. This study investigated the ~~standardly applied~~ radon spa therapy ~~in different~~commonly applied in various health ~~resorts compared to~~spas in comparison with a control intervention ~~in~~for rheumatic ~~out-patients~~outpatients and its results suggested beneficial analgesic effects of this therapy in rheumatic diseases for up to 9 months post-intervention (4). These observations were also confirmed by the ~~here presented~~ explorative RAD-ON01 study with 100 patients~~.~~ presented here. Nevertheless, ~~until today no~~ sufficient evidence of the beneficial effects of radon spa treatments ~~exist~~has not yet been provided, mostly due to the poor quality of many of the studies performed ~~studies (discussed in~~ (~~24~~26)~~)~~.

Prior to execution of the ~~presented~~ RAD-ON01 study presented, we hypothesized that the low doses of ~~emitted~~ alpha radiation emitted by radon might affect the immune system and thereby contribute to a reduction in or even resolution of ~~a~~ chronic inflammation as the main cause of painful degenerative diseases. Since the blood functions as a means of transport for immune cells to reach their target tissue, one would expect that feasible immune modulation properties of radon could be detected not only in the inflamed tissues but also in the peripheral blood. We expected that these small doses of alpha radiation as applied in ~~a~~ radon spa therapy ~~to~~ induce ~~rather~~ small alterations ~~of~~to the immune status. Now, for the first time, the RAD-ON01 trial has demonstrated a modulation of the peripheral immune system ~~by a~~through standard radon spa therapy (Figures 2-4). These modulations might contribute to the pain relieving effects of the therapy, but ~~the final~~conclusive proof of the ~~mechanistic basis~~underlying mechanisms still remains a challenge.

Neutrophils play a major role in ~~induction~~inducing and ~~maintenance of an~~maintaining inflammation. We found that the number of these innate immune cells temporarily decreased after radon spa ~~that might~~therapy, which may indicate ~~a~~ reduced tissue inflammation. Simultaneously, the number of eosinophils was elevated. Eosinophils are commonly viewed as non-specific destructive

effector cells ~~with~~which play a major ~~roles~~role in ~~allergy~~allergies and ~~parasite~~parasitic infections. ~~But nowadays~~However, it is now assumed that eosinophils have regulatory functions in tissue homeostasis and repair mechanisms ~~(discussed in~~ (~~25, 26~~27, 28)~~).~~. One might speculate that an elevation of eosinophils could contribute to ~~restoring~~the restoration of tissue homeostasis in chronically inflamed tissues.

~~Furthermore, we found the DCs to be temporarily elevated. These highly efficient antigen presenting cells circulate the periphery constantly capturing antigens and presenting these to T and B cells (27). The DCs express various pattern recognition receptors (PRRs) for classification of the captured antigens and depending on it their presentation is accompanied by either stimulatory or anti-inflammatory signals for T and B cells (27). Consequently, either adaptive immune responses or immune tolerance are induced, making DCs to important regulators of the immune system. The blood DCs are differentiated into mDCs and pDCs and both types were temporarily increased after the radon spa therapy. The mDCs express many different PRRs responding on several stimuli (27), but in contrast, the pDCs are more specialized on sensing nuclear acids and rather fostering pro-inflammatory responses (28). A temporary increase of DCs, as observed in the RAD-ON01 study, might indicate a rise of these effective regulators for an active suppression or resolution of the chronic inflammation and again for restoring tissue homeostasis.~~

Furthermore, we found the DCs to be temporarily elevated. These highly efficient antigen-presenting cells circulate through the periphery constantly, capturing antigens and presenting them to T and B cells (29). The DCs express various pattern recognition receptors (PRRs) for classification of the captured antigens and, depending on this, their presentation is accompanied by either stimulatory or anti-inflammatory signals for T and B cells (29). Consequently, either adaptive immune responses or immune tolerance are induced, making DCs important regulators of the immune system. The blood DCs are differentiated into mDCs and pDCs, and both types were temporarily increased after the radon spa therapy. The mDCs express many different PRRs responding to several stimuli (29) while, in contrast, the pDCs are more specialized in sensing nuclear acids and fostering pro-inflammatory responses (30). A temporary increase in DCs, as observed in the RAD-ON01 study, may indicate a rise in the number of these effective regulators for active suppression or resolution of chronic inflammation and again for restoration of tissue homeostasis.

Regarding the main immune cells, we also found the number of T cells and monocytes to be slightly, but long ~~lasting~~ lastingly increased. This also applied to their expression of HLA-DR, which belongs to the MHC class II complex and is generally expressed on professional antigen- presenting cells to show their captured antigens to T or B cells. Still, a few T cells express HLA-DR upon activation (~~29~~31) and these cells ~~were described~~have been found to capture and present autoantigens to other T cells and thus actively suppress them (~~30~~32). In contrast to ~~the~~ T cells, nearly all monocytes express HLA-DR, but ~~however~~ an elevated expression level ~~was related~~has been linked to a better therapy outcome in severe systemic inflammatory diseases (~~31, 32~~33, 34). Thus, an upregulation of HLA-DR on both cell types after radon exposure might actively contribute to ~~resolve~~resolving chronic ~~inflammations~~inflammation.

We ~~further~~also detected modulations within the T cell subsets. Interestingly, no effects on the composition of T_H or its subsets were detected despite their central role in the coordination of innate and adaptive immune cells. Only the T_{REGs} ~~were modulated~~, which are able to directly suppress the activation of other immune cells and ~~thus are even when occurring in a very low frequency~~are thus highly efficient and potent immune regulators~~.~~ even when occurring at very low frequency, were modulated. They play a significant role in the prevention of autoimmune diseases ~~(33).~~ ~~Thus,~~(35). Even tiny increases as observed here could ~~already~~ evoke potent local or systemic immune ~~suppressions~~suppression.

In contrast to ~~the~~ T_H, we detected a late decrease ~~of~~in T_C and different shifts within its subsets from week 12 ~~on~~onwards. The main function of T_C is the cytotoxic destruction of infected or degenerated host cells. Therefore, the MHC class I complexes, which are expressed by all nucleated host cells and continuously present intracellular peptides, are screened for foreign (e.g. viral) or degenerated molecules (e.g. tumor proteins). Since these MHC class I molecules are expressed by nearly all host cells, defects in their recognition ~~might~~may cause extensive tissue ~~damages~~damage. Indeed, a subgroup of $CD57^+$ T_C has already been shown to be elevated in patients with rheumatoid

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arthritis (~~34~~36). However, the role of T_C in autoimmune diseases is still unclear and controversial ~~(discussed in (35))~~(37), and animal studies on CD8 and CD4 deficient mice have suggested ~~rather~~that T_C may instead have a regulatory or even protective function ~~for the T_C~~ in arthritis (~~36~~38). In the ~~here presented~~ RAD-ON01 study presented here, we observed ~~the~~that T_C ~~to decrease~~decreased in the peripheral blood following the radon treatment, but further subtyping revealed shifts within the T_C subsets in the direction of effector subsets, suggesting an active contribution of ~~the~~ T_C. ~~In sum~~

To summarize, we observed a late decrease of naive and central memory T_C in favor of effector and effector memory T_C. In general, the first two circulate through the body or reside in tissues waiting ~~for the~~to encounter ~~of~~ their specific antigen, but lack ~~of~~ inflammatory and cytotoxic functions ~~(37).~~(39). Then~~,~~ upon challenge they proliferate extensively and generate effector or effector memory cells which then migrate into the target tissues for elimination of infected or degenerated cells accompanied by secretion of cytokines and chemokines ~~(38).~~(40). If available, the response of memory cells is much faster ~~compared to~~than that of naïve T cells ~~(37)~~(39) and ~~in fact we~~it was observed that the effector memory cells ~~increasing~~increased earlier than the effector cells. However ~~with about~~, occurring after around 12 weeks, all these effects appeared relatively late after radiation exposure, indicating that this response ~~to be in~~was a consequence of a previous occasion which was directly caused by radiation.

Lastly, shifts within the subsets of the innate NK cells were found even though their total number remained unaffected. The main cytotoxic NK1 subset (~~39~~41) increased shortly after radon spa therapy and simultaneously the $CD56^{+}/CD16^{-}$ NK3 subset, which has not yet been functionally ~~has not been~~ characterized ~~yet~~, decreased. Unlike this early modulation, the $CD56^{hi}/CD16^{-}$ NK2 subset, which has a regulatory function and primarily supports other immune cells by cytokine secretion (e.g. IFNγ, TNFα, GM-CSF, IL-10, IL-13) (~~40~~42), decreased continuously. However, we did not find any effects of the radon spa therapy on the expression of activating (NKG2c) or suppressing

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(NKG2a) molecules by NK cells (not shown). Thus, the role of ~~the~~ NK cells remains elusive. However, this innate immune response might pave the way for the delayed T_C response.

In conclusion, the ~~here presented~~ prospective and explorative RAD-~~ON-01~~ON01 study shows for the first time that immune modulations that may favor the attenuation of inflammation occur in the peripheral blood following radon spa therapy. ~~Since these observed modulations favor the attenuation of an inflammation, one~~One might speculate that radon and its secondary products might contribute to the restoration of balance in chronically dysregulated inflammatory tissues ~~which mainly caused the~~. The latter are a major cause of clinical symptoms such as pain or joint stiffness. ~~Hereby, certain~~Certain immune modulations occurred ~~already~~only shortly after the end of therapy. However, some effects, in particular shifts within the T_C subsets, appeared later or remained for a long time ~~pointing out~~, indicating secondary radiation effects. ~~Such secondary effects, including the~~The deactivation of immune cells as observed in the reduced CD69 expression on T, B and NK cells~~, might~~ may be responsible for the long-term improvement ~~of the~~in clinical symptoms which ~~were~~was reported by the majority of patients.

A recently published randomized, placebo-controlled intervention study showed that ~~both~~ exercise both with and without low-dose radon hyperthermia balneo treatment ~~impacted on~~affected bone metabolism and quality of life in a study population of an age group at risk ~~for~~of developing osteoporosis (~~41~~43). However, the patients ~~of~~in the therapy group had a slightly stronger reduction ~~of~~in the osteoclast-~~-~~stimulating protein receptor activator of the nuclear κB-ligand (~~41~~43). This study nicely illustrates the need ~~of~~for further randomized trials to ~~reveal~~investigate the effects of low doses of radon on the human body. As mentioned above, a RAD-ON02 study with a cross-over design is on the way. Taken together, ~~both~~the available studies expand the modes of action of radon to immune modulations and beneficial potential on bone metabolism. ~~Most likely~~It is highly probable that osteo-immunological mechanisms ~~(42) might be~~ (44) are influenced by ~~the~~ radon spa therapy.

~~Geological~~Location information

All authors are located in Germany. The entire study was ~~completely performed~~conducted in Bad Steben, which is located in Bavaria in southern Germany. All blood investigations were performed in Erlangen, which is also ~~is~~ located in Bavaria in southern Germany.

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Disclosure statement

The authors have nothing to declare.

References

1. Sokoloff, N. 1898. Röntgenstrahlen gegen Gelenkrheumatismus. *Wien Med Wochenschr* 12.
2. Franke, A., L. Reiner, H. G. Pratzel, T. Franke, and K. L. Resch. 2000. Long-term efficacy of radon spa therapy in rheumatoid arthritis--a randomized, sham-controlled study and follow-up. *Rheumatology (Oxford, England)* 39: 894-902.
3. Falkenbach, A., J. Kovacs, A. Franke, K. Jorgens, and K. Ammer. 2005. Radon therapy for the treatment of rheumatic diseases--review and meta-analysis of controlled clinical trials. *Rheumatology international* 25: 205-210.
4. Franke, A., and T. Franke. 2013. Long-term benefits of radon spa therapy in rheumatic diseases: results of the randomised, multi-centre IMuRa trial. *Rheumatology international* 33: 2839-2850.
5. Ott, O. J., C. Jeremias, U. S. Gaipl, B. Frey, M. Schmidt, and R. Fietkau. 2015. Radiotherapy for benign achillodynia : Long-term results of the Erlangen Dose Optimization Trial. *Strahlentherapie und Onkologie : Organ der Deutschen Rontgengesellschaft ... [et al]* 191: 979-984.
6. Ott, O. J., C. Jeremias, U. S. Gaipl, B. Frey, M. Schmidt, and R. Fietkau. 2014. Radiotherapy for benign calcaneodynia: long-term results of the Erlangen Dose Optimization (EDO) trial. *Strahlentherapie und Onkologie : Organ der Deutschen Rontgengesellschaft ... [et al]* 190: 671-675.

7. Ott, O. J., S. Hertel, U. S. Gaipl, B. Frey, M. Schmidt, and R. Fietkau. 2014. The Erlangen Dose Optimization Trial for radiotherapy of benign painful shoulder syndrome. Long-term results. *Strahlentherapie und Onkologie : Organ der Deutschen Rontgengesellschaft ... [et al]* 190: 394-398.
8. Ott, O. J., S. Hertel, U. S. Gaipl, B. Frey, M. Schmidt, and R. Fietkau. 2014. The Erlangen Dose Optimization trial for low-dose radiotherapy of benign painful elbow syndrome. Long-term results. *Strahlentherapie und Onkologie : Organ der Deutschen Rontgengesellschaft ... [et al]* 190: 293-297.
9. Trott, K. R., and F. Kamprad. 1999. Radiobiological mechanisms of anti-inflammatory radiotherapy. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology* 51: 197-203.
10. Frey, B., S. Hehlgans, F. Rodel, and U. S. Gaipl. 2015. Modulation of inflammation by low and high doses of ionizing radiation: Implications for benign and malign diseases. *Cancer letters* 368: 230-237.
11. Manda, K., A. Glasow, D. Paape, and G. Hildebrandt. 2012. Effects of ionizing radiation on the immune system with special emphasis on the interaction of dendritic and T cells. *Frontiers in oncology* 2: 102.
12. Wunderlich, R., A. Ernst, F. Rodel, R. Fietkau, O. Ott, K. Lauber, B. Frey, and U. S. Gaipl. 2015. Low and moderate doses of ionizing radiation up to 2 Gy modulate transmigration and chemotaxis of activated macrophages, provoke an anti-inflammatory cytokine milieu, but do not impact upon viability and phagocytic function. *Clinical and experimental immunology* 179: 50-61.
13. Frischholz, B., R. Wunderlich, P. F. Ruhle, C. Schorn, F. Rodel, L. Keilholz, R. Fietkau, U. S. Gaipl, and B. Frey. 2013. Reduced secretion of the inflammatory cytokine IL-1beta by stimulated peritoneal macrophages of radiosensitive Balb/c mice after exposure to 0.5 or 0.7 Gy of ionizing radiation. *Autoimmunity* 46: 323-328.
14. Large, M., S. Hehlgans, S. Reichert, U. S. Gaipl, C. Fournier, C. Rodel, C. Weiss, and F. Rodel. 2015. Study of the anti-inflammatory effects of low-dose radiation: The contribution of biphasic regulation of the antioxidative system in endothelial cells. *Strahlentherapie und Onkologie : Organ der Deutschen Rontgengesellschaft ... [et al]* 191: 742-749.
15. Rodel, F., L. Keilholz, M. Herrmann, R. Sauer, and G. Hildebrandt. 2007. Radiobiological mechanisms in inflammatory diseases of low-dose radiation therapy. *International journal of radiation biology* 83: 357-366.
16. Gaipl, U. S., S. Meister, B. Lodermann, F. Rodel, R. Fietkau, M. Herrmann, P. M. Kern, and B. Frey. 2009. Activation-induced cell death and total Akt content of granulocytes show a biphasic course after low-dose radiation. *Autoimmunity* 42: 340-342.
17. Kern, P. M., and L. Keilholz. 2009. Radio-immunological mechanisms of anti-inflammatory treatment: is there a way from the past into the future? *Autoimmunity* 42: 337-339.
18. Niewald, M., M. H. Seegenschmiedt, O. Micke, S. Graeber, R. Muecke, V. Schaefer, C. Scheid, J. Fleckenstein, N. Licht, and C. Ruebe. 2012. Randomized, multicenter trial on the effect of radiation therapy on plantar fasciitis (painful heel spur) comparing a standard dose with a very low dose: mature results after 12 months' follow-up. *International journal of radiation oncology, biology, physics* 84: e455-462.
19. Kataoka, T. 2013. Study of antioxidative effects and anti-inflammatory effects in mice due to low-dose X-irradiation or radon inhalation. *Journal of radiation research* 54: 587-596.
20. Zdrojewicz, Z., and J. J. Strzelczyk. 2006. Radon treatment controversy. *Dose-response : a publication of International Hormesis Society* 4: 106-118.
21. Yamaoka, K., F. Mitsunobu, K. Hanamoto, S. Mori, Y. Tanizaki, and K. Sugita. 2004. Study on biologic effects of radon and thermal therapy on osteoarthritis. *The journal of pain : official journal of the American Pain Society* 5: 20-25.

22. Salomaa, S., K. M. Prise, M. J. Atkinson, A. Wojcik, A. Auvinen, B. Grosche, L. Sabatier, J. R. Jourdain, E. Salminen, S. Baatout, U. Kulka, H. Rabus, E. Blanchardon, D. Averbeck, and W. Weiss. 2013. State of the art in research into the risk of low dose radiation exposure--findings of the fourth MELODI workshop. *Journal of radiological protection : official journal of the Society for Radiological Protection* 33: 589-603.

23. ~~Pratzel, H. G., and P. Deetjen. 1997. *Radon in der Kurortmedizin*. ISMH Verlag, Geretsried~~Ruhle, P. F., R. Fietkau, U. S. Gaipl, and B. Frey. 2016. Development of a Modular Assay for Detailed Immunophenotyping of Peripheral Human Whole Blood Samples by Multicolor Flow Cytometry. *International journal of molecular sciences* 17.

~~24.~~24. Pratzel, H. G., and P. Deetjen. 1997. *Radon in der Kurortmedizin*. ISMH Verlag, Geretsried.

25. Deetjen, P., A. Falkenbach, D. Harder, H. Jöckel, A. Kaul, and H. von Philipsborn. 2005. *Radon als Heilmittel, Therapeutische Wirksamkeit, biologischer Wirkungsmechanismus und vergleichende Risikobewertung*. Verlag Dr. Kovac, Hamburg.

26. Santos, I., P. Cantista, and C. Vasconcelos. 2015. Balneotherapy in rheumatoid arthritis-a systematic review. *International journal of biometeorology*.

~~25~~27. Jacobsen, E. A., R. A. Helmers, J. J. Lee, and N. A. Lee. 2012. The expanding role(s) of eosinophils in health and disease. *Blood* 120: 3882-3890.

~~26~~28. Furuta, G. T., F. D. Atkins, N. A. Lee, and J. J. Lee. 2014. Changing roles of eosinophils in health and disease. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology* 113: 3-8.

~~27~~29. Zanoni, I., and F. Granucci. 2010. Regulation of antigen uptake, migration, and lifespan of dendritic cell by Toll-like receptors. *Journal of molecular medicine (Berlin, Germany)* 88: 873-880.

~~28~~30. Reizis, B., A. Bunin, H. S. Ghosh, K. L. Lewis, and V. Sisirak. 2011. Plasmacytoid dendritic cells: recent progress and open questions. *Annual review of immunology* 29: 163-183.

~~29~~31. Salgado, F. J., J. Lojo, C. M. Fernandez-Alonso, J. Vinuela, O. J. Cordero, and M. Nogueira. 2002. Interleukin-dependent modulation of HLA-DR expression on CD4and CD8 activated T cells. *Immunology and cell biology* 80: 138-147.

~~30~~32. LaSalle, J. M., K. Ota, and D. A. Hafler. 1991. Presentation of autoantigen by human T cells. *Journal of immunology (Baltimore, Md. : 1950)* 147: 774-780.

~~31~~33. Antoniades, C. G., P. A. Berry, E. T. Davies, M. Hussain, W. Bernal, D. Vergani, and J. Wendon. 2006. Reduced monocyte HLA-DR expression: a novel biomarker of disease severity and outcome in acetaminophen-induced acute liver failure. *Hepatology* 44: 34-43.

~~32~~34. Cheadle, W. G., M. J. Hershman, S. R. Wellhausen, and H. C. Polk, Jr. 1991. HLA-DR antigen expression on peripheral blood monocytes correlates with surgical infection. *American journal of surgery* 161: 639-645.

~~33~~35. Buckner, J. H. 2010. Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory T cells in human autoimmune diseases. *Nature reviews. Immunology* 10: 849-859.

~~34~~36. Arai, K., S. Yamamura, S. Seki, T. Hanyu, H. E. Takahashi, and T. Abo. 1998. Increase of CD57+ T cells in knee joints and adjacent bone marrow of rheumatoid arthritis (RA) patients: implication for an anti-inflammatory role. *Clinical and experimental immunology* 111: 345-352.

~~35~~37. Alzabin, S., and R. O. Williams. 2011. Effector T cells in rheumatoid arthritis: Lessons from animal models. *Febs Lett* 585: 3649-3659.

~~36~~38. Taneja, V., N. Taneja, T. Paisansinsup, M. Behrens, M. Griffiths, H. Luthra, and C. S. David. 2002. CD4 and CD8 T cells in susceptibility/protection to collagen-induced arthritis in HLA-DQ8-transgenic mice: implications for rheumatoid arthritis. *Journal of immunology (Baltimore, Md. : 1950)* 168: 5867-5875.

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~~37~~39. Lanzavecchia, A., and F. Sallusto. 2005. Understanding the generation and function of memory T cell subsets. *Current opinion in immunology* 17: 326-332.

~~38~~40. Mittrucker, H. W., A. Visekruna, and M. Huber. 2014. Heterogeneity in the Differentiation and Function of CD8(+) T Cells. *Arch Immunol Ther Ex* 62: 449-458.

~~39~~41. Jacobs, R., G. Hintzen, A. Kemper, K. Beul, S. Kempf, G. Behrens, K. W. Sykora, and R. E. Schmidt. 2001. CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells. *European journal of immunology* 31: 3121-3127.

~~40~~42. Cooper, M. A., T. A. Fehniger, S. C. Turner, K. S. Chen, B. A. Ghaheri, T. Ghayur, W. E. Carson, and M. A. Caligiuri. 2001. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood* 97: 3146-3151.

~~41~~43. Winklmayr, M., C. Kluge, W. Winklmayr, H. Kuchenhoff, M. Steiner, M. Ritter, and A. Hartl. 2015. Radon balneotherapy and physical activity for osteoporosis prevention: a randomized, placebo-controlled intervention study. *Radiation and environmental biophysics* 54: 123-136.

~~42~~44. Jimi, E., and S. Ghosh. 2005. Role of nuclear factor-kappaB in the immune system and bone. *Immunological reviews* 208: 80-87.

Table 1. Patient characteristics ~~at start of the radon spa therapy~~.

Patient [number]	
In total	~~101~~100
Sex [number]	
Male	40
Female	~~61~~60
Age [years]	
Mean	60.2
Range	28 – 75
Diagnosis [number]	
Spine only	~~39~~38
Joints only	32
Spine and joints	22
Fibromyalgia	8

Legends to the figures

Figure 1: Long-lasting pain reduction ~~of pain~~ following ~~the~~ radon spa therapy. ~~The~~100 patients determined their pain perception on a VAS ranging from 0 (no pain) to 10 (worst imaginable pain) on-site ~~at all examination time points~~ (**A**) as well as retrospectively in week 30 (**B**). ~~In both cases a significant pain reduction was observed. It was estimated even higher when evaluated retrospectively, as here a higher starting value (week 0) was indicated (**B**). Such decrease in pain was indicated by the majority~~ 69% of patients ~~whereby 69%~~ had a long~~-~~lasting (**dark grey bar**) and 18% a sustained (**light grey bar**) effect (**C**). ~~Of all patients, 13% had no beneficial therapy effect on their pain perception (**white bar**).~~ These subjective impressions were confirmed by pressure point measurements ~~determining the personal pain sensitivity~~ at previously defined trigger points~~.~~ (dolorimetry). The pressure required ~~pressure~~ to induce pain increased steadily for both~~,~~ the mean of all 8 trigger points (**white circle**) ~~as well as that~~and the one located in the most affected area (**black circle**) (**D**). Box~~-whiskers~~ and whisker plots (A, B), bar chart (C) or mean ± SEM (D). n = 100. ~~X-axis represents weeks after the start of therapy (A,B,D).~~ All statistical analyses were performed ~~versus~~vs. week 0 (before start of therapy) using the Friedman test with Wilcoxon correction (A,B), ANOVA (~~C~~D: max. pressure point) or ANOVA with repeated measurements (~~C~~D: mean pressure points~~);~~). VAS: visual analogue scale; *: p<0.05, **: p<0.01; ***: p<0.001.

Figure 2: Minor modulations of the major immune cells by radon spa therapy. The T~~-~~cells (**A**) and monocytes (**D**) were slightly but long~~-lasting~~lastingly increased after radon spa therapy. The B cells (**B**) and NK cells (**C**) were not affected. Regulatory DCs which were identified as mDCs (**E**) and pDCs (**F**) were temporarily increased shortly after therapy. Likewise, the neutrophils (**G**) and eosinophils (**H**) were affected shortly after therapy. ~~Box-whiskers-plots. X-axis represents weeks after the start of therapy.~~Box and whisker plots. All statistical analyses were performed vs. time point 0 (before therapy) using the paired t test. *: p<0.05, **: p<0.01; ***: p<0.001.

Figure 3: Modifications in several immune cell subsets by radon spa therapy. ~~The immunophenotyping assay identified 40 different~~Many immune cell subsets~~, whereby most of them~~ were not affected by radon spa therapy~~. These included the~~, for example T_H (**A**~~),~~) and most of its subsets such as ~~the~~ T_H1 (**B**) or T_H2 (**C**~~). Of the T_H,~~); only ~~the immunosuppressive~~ T_{REGs} were affected and elevated for up to 12 weeks ~~after the first contact of the patients with radon~~ (**D**). In contrast to ~~the~~ T_H, ~~the~~ T_C ~~were~~was affected by the therapy and decreased from week 12 on (**E**). Moreover, T_C subsets revealed shifts from naïve (**F**) and central memory (**H**) subsets ~~into~~in the direction of the cytotoxic effector (**G**) and effector memory cells (**I**). Like the general T_C decrease, these shifts

occurred relatively late after therapy. Furthermore, different shifts within the NK cell subsets were ~~foremost~~ mainly identified shortly after therapy ~~identified~~ (**J**~~-~~**L**). Box ~~whiskers~~ and whisker plots. ~~X-axis represents weeks after the start of therapy.~~ All statistical analyses were performed vs. time point 0 (before therapy) using the paired t test. *: p<0.05, **: p<0.01; ***: p<0.001.

Figure 4: Major changes in the activation state of lymphocytes by radon spa therapy. The expression of the common activation CD69 was strongly reduced on T cells (**A**), B cells (**B**) and NK cells (**C**) after radon spa therapy. In contrast, ~~the~~ HLA-DR expression was elevated on T cells (**D**), but differed between T_H (**E**) and T_C (**F**). ~~Box whiskers plots. X axis represents weeks after the start of therapy.~~Box and whisker plots. All statistical analyses were performed vs. time point 0 (before therapy) using the paired t test. *: p<0.05, **: p<0.01; ***: p<0.001.

Appendices

Supplementary Table S1: List of Antibodies.

Specificity	Clone	Fluorochrome	Amount per 100 µl blood	Vendor
CD1c	L161	APC	1,0	eBioscience
CD1d	CD1d42	PE	2,0	BD Biosciences
CD3	UCHT1	KO	3,0	Beckman Coulter
CD3	UCHT1	V450	1,0	BD Biosciences
CD3	UCHT1	FITC	3,0	BD Biosciences
CD4	RPA-T4	PCC5.5	1,0	BD Biosciences
CD5	UCHT2	PECy7	0,5	eBioscience
CD8	HIT8a	FITC	4,0	BD Biosciences
CD11c	B-ly6	V450	1,0	BD Biosciences
CD14	RMO52	FITC	10,0	Beckman Coulter
CD14	M5E2	FITC	4,0	BD Biosciences
CD16	B73.1	PE	2,5	BD Biosciences
CD16	B73.1	FITC	2,5	BD Biosciences
CD16	3G8	KO	2,0	Beckman Coulter
CD19	J3-119	KO	1,0	Beckman Coulter
CD19	HIB19	FITC	2,0	BD Biosciences
CD20	2H7	FITC	4,0	BD Biosciences
CD24	ML5	PCC5.5	2,5	BD Biosciences
CD25	M-A251	BV421	2,5	BD Biosciences
CD25	M-A251	PECy7	5,0	BD Biosciences
CD25	M-A251	PE	4,0	BD Biosciences
CD27	M-T271	V450	1,0	BD Biosciences
CD34	563	APC	5,0	BD Biosciences
CD38	HIT2	APC	5,0	BD Biosciences
CD45	HI30	PECy7	1,0	BD Biosciences
CD45RA	HI100	PECy7	2,0	BD Biosciences

Specificity	Clone	Fluorochrome	Amount per 100 µl blood	Vendor
CD56	B159	FITC	1,0	BD Biosciences
CD56	B159	PCC5.5	4,0	BD Biosciences
CD64	10.1	V450	2,5	BD Biosciences
CD66	B1.1/CD66	FITC	2,0	BD Biosciences
CD69	FN50	PEV770	2,0	Miltenyi Biotec
CD69	FN50	FITC	2,0	Miltenyi Biotec
CD80	2D10	APC	2,0	Miltenyi Biotec
CD80	L307.4	APCH7	5,0	BD Biosciences
CD83	HB15e	PECy7	2,5	eBioscience
CD86	IT2.2	PCC5.5	5,0	BioLegend
CD94	REA113	PEV770	2,0	Miltenyi Biotec
CD123	7G3	PCC5.5	7,0	BD Biosciences
CD127	HIL-7R-M21	FITC	20,0	BD Biosciences
CD133/1	AC133	PE	5,0	Miltenyi Biotec
CD146	P1H12	FITC	2,5	BD Biosciences
CD152	BNI3	APC	10,0	BD Biosciences
CD159a	131411	APC	10,0	R&D Systems
CD159c	134591	A488	10,0	R&D Systems
CD183	1C6/CXCR3	APC	3,0	BD Biosciences
CD196	11A9	PE	4,0	BD Biosciences
CD197	150503	PE	5,0	BD Biosciences
CD274	MIH1	PE	20,0	BD Biosciences
CD279	MIH4	APC	10,0	BD Biosciences
CD314	149810	APC	2,0	R&D Systems
HLA-DR	Immu-357	KO	2,0	Beckman Coulter
TCRab	T10B9.1A-31	PE	20,0	BD Biosciences
TCRgd	11F2	FITC	10,0	BD Biosciences

Abbreviations used for fluorochromes: FITC: Fluorescein isothiocyanate; A488: Alexa-488; PE: Phycoerythrin; PCC5.5: PerCp-Cy5.5; PECy7: PE-Cy7; PEV770: PE-Vio770; APC: Allophycocyanin; APCH7: APC-H7; V450: Horizon V450; BV421: Brilliant Violet 421; KO: Krome Orange

Supplementary Table S2: Overview of staining panels.

	Blue: 488nm				Red: 638nm		Violet: 405nm	
	525/40 FITC|A488	575/30 PE	695/30 PCC5.5	755/LP PECy7| PEV770	660/20 APC	755/LP APCH7	450/50 V450|BV421	550/40 KO
P01	CD8	CD197	CD4	CD45RA	CD38		CD3	
P02	CD127	CD196	CD4	CD25	CD183		CD3	
P03	TCRγ/δ	TCRα/β			CD152		CD3	
P04	CD20		CD24	CD5	CD38		CD27	CD19
P05	CD19/20	CD25	CD86	CD69	CD279	CD80	CD3	HLA-DR
P06	CD69	CD16	CD56		CD314		CD25	CD3
P07	CD159c	CD16	CD56	CD94	CD159a			CD3
P08	CD14	CD16	CD86		CD80		CD64	HLA-DR
P09	CD66						CD64	CD16
P10	LIN[1]	CD274	CD123	CD83	CD1c		CD11c	HLA-DR
P11	CD146	CD133.1		CD45	CD34			

Abbreviations used for fluorochromes: FITC: Fluorescein isothiocyanate; A488: Alexa-488; PE: Phycoerythrin; PCC5.5: PerCp-Cy5.5; PECy7: PE-Cy7; PEV770: PE-Vio770; APC: Allophycocyanin; APCH7: APC-H7; V450: Horizon V450; BV421: Brilliant Violet 421; KO: Krome Orange

Supplementary Table S3: Cytometer settings.

Laser	Power	Detector	Voltage	Gain	Filter	Range	Fluorochrome
488 nm	22 mW	FSC	300	5.0	–	–	–
		SSC	300	2.0	–	–	–
		FL1	470	1	525 BP 40	505 - 545	FITC, Alexa 488
		FL2	500	1	575 BP 30	560 - 590	PE
		FL3	430	1	620 BP 30	605 - 635	PI
		FL4	630	1	695 BP 30	680 - 710	PerCP Cy5.5
		FL5	600	1	755 LP	> 755	PE Cy7, PE Vio770
638 nm	25 mW	FL6	650	1	660 BP 20	650 - 670	APC
		FL7	250	1	725 BP 20	715 - 735	--
		FL8	600	1	755 LP	> 755	APC H7
405 nm	40 mW	FL9	470	1	450 BP 50	425 - 475	Horizon V450, Brilliant Violet 421
		FL10	400	1	550 BP 40	530 - 570	Krome Orange

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