Intraperitoneal Oxidative Stress in Rabbits with Papillomavirus-Associated Head and Neck Cancer Induces Tumoricidal Immune Response That Is Adoptively Transferable

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Abstract

Purpose: How tumors evade or suppress immune surveillance is a key question in cancer research, and overcoming immune escape is a major goal for lengthening remission after cancer treatment. Here, we used the papillomavirus-associated rabbit auricular VX2 carcinoma, a model for studying human head and neck cancer, to reveal the mechanisms underlying the antitumorigenic effects of intraperitoneal oxidative stress following O3/O2-pneumoperitoneum (O3/O2-PP) treatment.

Experimental Design: Solid auricular VX2 tumors were induced in immune-competent adult New Zealand White Rabbits. Animals were O3/O2-PP- or sham-treated, after which they underwent tumor ablation upon reaching no-go criteria. CD3+ tumor-infiltrating lymphocytes (TIL) were evaluated by immunohistochemistry, and expression levels of 84 immune response genes were measured by quantitative real-time PCR. Adoptive transfer of peripheral blood leukocytes (PBL)—derived from animals with tumor regression—into control animals with progressing tumors was implemented to assess acquired tumor resistance functionally.

Results: Auricular VX2 tumors regressing after O3/O2-PP treatment exhibited increased levels of CD3+ TILs; they also exhibited enhanced expression of genes that encode receptors involved in pattern recognition, molecules that are required for antigen presentation and T cell activation, and inflammatory mediators. Adoptive cell transfer of PBLs from donor rabbits with regressing tumors to recipient rabbits with newly implanted VX2 carcinoma resulted in acquired tumor resistance of the host and tumor regression.

Conclusion: Intraperitoneal oxidative stress effectively converts the immune response against the papillomavirus-associated rabbit VX2 carcinoma from tumor permissive to tumoricidal and leads to a sustainable, adoptively transferable oncolytic immune response. Clin Cancer Res; 20(16); 4289–301. ©2014 AACR.

Introduction

Treatment of cancer is one of the major challenges in medical care. Despite progress in surgery, radiotherapy, and chemotherapy, the prognosis for surviving aggressive cancer is often poor. Immunotherapies represent effective approaches to combat cancer by mobilizing components of the immune system for tumor destruction. For this, recombinant cytokines or monoclonal antibodies targeting tumor-specific antigens (TSA), and their receptors or ligands were all found to exert antitumoral effects. Vaccination against TSAs is used as a therapeutic approach in the clinic (reviewed in ref. 1). To design novel therapeutic interventions that stimulate tumoricidal immune responses more effectively, the specific hallmarks of cancer have to be taken into account (2). Thus, solid tumors consist of relatively few neoplastic cells (cancer stem cells, CSC), and numerous distinct cell types and subtypes within the associated stroma that collectively facilitate tumor growth and progression. A recently added hallmark of tumor pathogenesis is the deregulation of cellular energetics due to genomic instability and the active immune evasion of cancer cells enabled by tumor-mediated inflammation (2), described as cancer immunoediting (3). However, tumor-associated inflammation not only initiates and supports tumor development, but can also produce antitumorogenic immune responses leading to tumor regression. A meta-analysis of 52 human
Translational Relevance
After application of intraperitoneal oxidative stress, the observed regression of the rabbit auricular VX2 carcinoma—a model system for human head and neck cancer—suggested a role of the immune system in tumor clearance. Here, we demonstrate this effect to be dependent on immune cells, as the number of tumor-infiltrating lymphocytes and expression of immune-relevant genes significantly rose in remitting tumor tissues. Furthermore, adoptive transfer of peripheral blood leukocytes, derived from the same animals, could induce tumor regression in untreated animals with continuously growing tumors. Because the investigated VX2 carcinoma is papillomavirus-associated, studying the observed tumoricidal immune response in this model system carries great promise in identifying the mechanisms underlying the immune escape of HPV-positive human head and neck cancers, thereby paving the way for new therapeutic approaches.

Materials and Methods
Animals
Overall, 40 adult Iffa Credo NZW rabbits (Charles River WIGA) in a body weight (BW) range from 2.0 to 3.0 kg were used, 8 of them as donors of the VX2 cell suspension necessary for the induction of auricular tumors. Rabbits were kept in individual steel cages under standardized air conditioning at 20°C to 22°C, 50% to 60% humidity at a 12-hour artificial day/night rhythm and had access to food and water ad libitum. Animals could acclimatize for at least 7 days in the hutch before the experimental procedure was started. Signs of distress, pain, or cachexia—defined as weight loss above 20%—were criteria for euthanasia throughout the study. The animal experiments were approved by the regional board Giessen, Germany (V54-19c20-15(1) MR, Nr.34/ 2011), according to the German Animal Protection Law.

Experimental design
The study consisted of two consecutive experimental phases. In the first phase (O3/O2-PP therapy, Fig. 1A), a solid auricular VX2 tumor was induced in n = 20 animals. The tumor size was measured daily with a digital caliper and a peritoneal puncture but no gas insufflation (n = 20). Signs of distress, pain, or cachexia—defined as weight loss above 20%—were criteria for euthanasia throughout the study. The animal experiments were approved by the regional board Giessen, Germany (V54-19c20-15(1) MR, Nr.34/ 2011), according to the German Animal Protection Law.

Tumor transplantation/therapeutic approach
VX2 tumor cells from frozen stocks were propagated by repeated intramuscular passage within the quadriceps muscles of NZW rabbits. After three consecutive passages, VX2 tumor cell suspensions were generated from fragmented...
Figure 1. Experimental design and growth kinetics of VX2 tumors. A, treatment scheme of the O3/O2-PP therapy and the defined no-go criteria. B and C, growth kinetics of VX2 tumors in animals of the O3/O2-PP and sham group. Mean values ± SEM are shown if the number of samples was >3 (filled icons). If the number was ≤3, the moving average over 3 days is shown by dashed lines. The gray dashed line indicates the threshold of tumor volume at which the therapeutic approach started. D, Kaplan–Meier analysis depicting TTR probability and TTP probability of solid auricular VX2 tumors (E). Treatment of each individual animal started when the auricular VX2 tumor exceeded a volume of 2,500 mm³ and was set to day 0. The TTP or TTR probabilities were defined by the tumor volume. The TTP was reached when the tumor volume increased above 6,000 mm³ and TTR was reached when the tumor volume decreased below 25% of its maximal volume. Statistically significant changes between the O3/O2-PP and the sham group were calculated by the log-rank test; *, P < 0.05; numbers and percentages of tumors showing regression (B and D) or progression (C and E) are shown in brackets.
tumor tissues and used for induction of auricular VX2 tumors as described in ref. (9). Tumor volume was measured daily and growth was allowed until the solid auricular tumor had reached a volume of $>$2,500 mm$^3$. At this stage, a daily therapeutic treatment with O$_3$/O$_2$ gas mixture inflated into the peritoneal cavity (O$_3$/O$_2$-PP) or sham treatment (puncture only) were performed as described elsewhere (14).

**Surgical ablation of the tumor**

Once the no-go criteria defined by the size of the VX2 tumor were reached, the animal was sedated by Robiniul (0.1 mL/kg BW, Riemser Arzneimittel AG; s.c.) and anesthetized by a mixture of Rompun (5 mg/kg BW, Bayer Vital GmbH) / Ketavet (70 mg/kg BW, Pharmacia GmbH; i.m.). Suprarenin (1 mg/mL, Sanofi-Aventis) was subcutaneously injected peritumorally, the auricular artery was ligated proximal to the tumor, and the complete tumor removed off from the adventitia (Fig. 2B and C). The wound was subsequently bandaged under compression. Compresses were changed daily until the bleeding completely stopped. Analgesic treatment with Temgesic (Essex Pharma; s.c.) was maintained for at least 2 days, dependent on the stage of wound healing.

**Blood parameters**

Arterial blood samples from the auricular artery of the left tumor-free ear were taken before inoculation of the VX2 tumor cell suspension (base value) and, shortly before the first and directly after the last treatment within the 5-day period of O$_3$/O$_2$-PP or sham treatment. For hematologic investigations, an autoanalyzer (Vet abc Animal Blood Counter; ABX Diagnostics) was carefully adjusted and validated for the analysis of rabbit blood. Data were expressed as mean values ± SEM.

**PET–CT**

Rabbits were anesthetized by Robiniul plus Ketavet as described, and 0.14 μCi/kg BW radioactive labeled FDG (2-[18F]-fluoro-2-deoxy-D-glucose) diluted in 1 mL of physiologic saline solution was injected into the anterior vein of the ear contralateral to the tumor. A combined PET–CT analysis of the head and thorax was performed 30 minutes after FDG injection using an integrated PET–CT device (Biograph 6 Truepoint PET/CT; Siemens Healthcare). The PET–CT scans, data reconstruction, and analysis were performed according to previously described protocols (15).

**Adoptive PBL transfer**

Animals from experimental phase I were used as donors of PBLs at day 90 after VX2 tumor implantation. Blood from the lateral auricular vein was taken and PBLs were isolated by lysis of erythrocytes. For lysis, 3 mL of EDTA-blood was incubated at room temperature for 10 minutes in 90 mL of lysis buffer (1.55 mol/L NH$_4$Cl, 100 mmol/L KHCO$_3$, 10 mmol/L EDTA), after spinning down the cell pellets were washed twice in Ca$^{++}$/Mg$^{++}$-free PBS. For each transfer, $5 \times 10^6$ cells in 1 mL of a physiologic NaCl solution were injected into the lateral auricular vein of the left ear. PBLs from (i) 3 individual animals with tumor regression as a consequence of the O$_3$/O$_2$-PP therapy and, (ii) 3 individual animals that exhibited progressive metastasizing tumor growth due to ineffective sham treatment were transferred into two recipients per donor. In parallel, to the adoptive transfer of PBLs, VX2 tumor was induced in the contralateral ear as described in ref. (9). Body temperature of the recipients was measured before and the first 2 days after cell transfer using a rectal thermometer.

**RT$^2$ profiler PCR array**

Information about the expression level of genes corresponding to the rabbit innate and adaptive immune system was assessed by the RT$^2$ Profiler PCR Array (PANZ-052ZA-24 for rabbit; SABiosciences) using probes of isolated total RNA from $n = 6$ solid tumors under remission (induced by O$_3$/O$_2$-PP) and $n = 6$ tumors under progression (unaffected by sham treatment). For each tumor sample, one 96-well RT$^2$ Profiler PCR Array plate containing 84 target genes and five independent housekeeping genes was used. Total RNA was isolated using the RNasy Mini kit (Qiagen) and integrity, purity, and yield were analyzed with the Expertion automated electrophoresis system (Bio-Rad Laboratories). Single-strand cDNA from 0.5 μg total RNA was synthesized using the RT$^2$ First Strand Kit, and real-time PCR (ABI PRISM 7900HT System; Applied Biosystems) was performed according to the instructions in the RT$^2$ Profiler PCR Array manual. The $C_t$ values of each of the 84 target genes of a given plate were normalized with the average $C_t$ values of the five independent housekeeping genes included on the same assay plate. Mean values of fold changes in mRNA levels derived from solid tumors under remission compared with mRNA expression levels of tumors under progression were calculated.

**Immunohistochemistry and quantitative image analysis**

Formalin-fixed VX2 tumor tissue slices (7-μm thick) were immunostained with a mouse specific anti-CD3 antibody (Biozol; diluted 1:1,000) as described in ref. (16). To enhance the staining signals, the Tyramide Signal Amplification (TSA) System (PerkinElmer) was used according to the manufacturer’s protocol. Quantitative image analysis of immunostained CD3$^+$ T cells was performed using an Olympus AX70 microscope (Olympus Optical), including a SPOT RT Slider Camera (Diagnostic Instruments Inc.) and the MCID Image system (Imaging Research Inc.). The border of staining intensity for positive signals was defined and the number of CD3$^+$ spots was automatically counted. Staining signals with a size of <10 pixel were excluded as false-positive events. At least 10 tumor sections of each tumor, immunostained for CD3, were digitized, depending on the size of the tumor (small for tumors under remission). Data were calculated as number of CD3$^+$ T cells per square millimeter solid VX2 tumor tissue.
**Statistical analysis**

In all analyses, a *P* value < 0.05 was considered as significant. For calculation of statistical differences in mRNA expression levels measured by the RT² Profiler PCR Array, a paired *t* test was performed by using the free RT² Profiler PCR Array Data Analysis software provided by the manufacturer (http://www.sabiosciences.com/pctooldataanal.php). In all other cases, the GraphPad Prism Software 4.0 (GraphPad Software) was used. If a given mRNA quantified by the RT² Profiler PCR Array was induced only in one experimental group, a nonparametric two-tailed Mann–Whitney test of the ΔΔCt values was performed. The log-rank test was used for comparison of progression/regression rates between both experimental groups. To synchronize the course of tumor growth, the time point when the primary tumor reached 2,500 mm³ in size was set to day zero. The day of the final outcome was defined as the day a tumor had reached the no-go criteria for regression or progression. The unpaired two-tailed *t* test with Welch’s correction was used for comparison of changes in blood parameters directly before and after therapeutic intervention and for changes in the number of CD3⁺ T cells detected within the tumor tissue.
Results

O3/O2-PP therapy eradicates solid VX2 tumors at a high success rate

In the first experimental phase (O3/O2-PP therapy, experimental design is shown in Fig. 1A), VX2 tumor cell suspension was injected subcutaneously in 20 NZW rabbits, which resulted in the development of a solid auricular VX2 tumor in 18 animals (tumor take-rate 90%), reaching a volume of 2,500 mm$^3$ at day 13 ± 2 (Fig. 1B and C). At this stage, animals received either the O3/O2-PP therapy or underwent sham treatment. Tumor volume further increased until around day 18, independent of the treatment. Thereafter, in 7 out of 10 animals (70%) that had received O3/O2-PP treatment, the tumor continuously decreased (Fig. 1B), whereas in 6 out of 8 sham-treated animals (75%), tumor size further increased above a critical volume of 6,000 mm$^3$ (Fig. 1C). At this stage, tumors strongly ulcerate, which finally leads to death. Kaplan-Meier analysis revealed that the time to tumor regression (TTR) probability was significantly higher after O3/O2-PP therapy in comparison with sham-treated animals (Fig. 1D). Accordingly, sham treatment had no impact on tumor development. The time to tumor progression (TTP) probability was significantly higher in the sham compared with the O3/O2-PP–treated group (Fig. 1E). Nevertheless, spontaneous remission occurred in 2 out of 8 sham-treated animals (25%) albeit the period required for spontaneous tumor clearance was longer (19 ± 2 days) in comparison with the time needed for O3/O2-PP–induced remission (14 ± 2 days).

According to the typical course of HNSCC disease, all animals exhibiting progression of the primary tumor (n = 3 for O3/O2-PP treatment and n = 6 for sham treatment) demonstrated metastases in the ipsilateral lymph nodes (Fig. 2A). Nearly all animals that had cleared the primary tumor were free of lymph node metastases (n = 6 for O3/O2-PP treatment and n = 2 for sham treatment) except 1 animal from the O3/O2-PP group (Fig. 2A). Presence of metastases in cervical lymph nodes was diagnosed by PET-CT (Fig. 2E–L) in the living animal (Fig. 2E–I) and by macroscopic (Fig. 2B) and histologic postmortem examination (Fig. 2K–L). In addition, within the 90-day observation period, the therapeutic effect of O3/O2-PP suggests protection against metastases.

O3/O2-PP–induced tumor clearance is associated with an increase in white blood cells and TILs

To test the hypothesis that leukocytes are involved in the O3/O2-PP–induced tumor defense, we analyzed major blood parameters before and directly after the last therapeutic intervention. The hemogram revealed a significant increase in white blood cells (WBC) exclusively in O3/O2-PP–treated animals (Fig. 3A), whereas other blood values remained within the physiologic range (Supplementary Table S1). Quantitative image analysis exhibited a significant 5-fold increase in the number of CD3$^+$T lymphocytes in the tissue of tumors that underwent remission as compared with the tissue of tumors that exhibited progression (Fig. 3B–D). This suggests that TILs are causally involved in VX2 tumor clearance.

Immune signature in tumors under O3/O2-PP–induced remission

A multiplex qPCR Assay representing 84 different inflammation relevant markers was used to decipher immune reactions occurring in tumors remitting after O3/O2-PP treatment. The entire list of all components can be found in Supplementary Table S2.

Tumor remission is associated with enhanced transcription of immune-related genes in the respective tumor tissue, which includes pattern recognition receptors (PRR; Fig. 4A), elements functionally involved in signal transduction cascades (Fig. 4B), and factors known to modulate, regulate, or support ongoing immune responses by different mechanisms (Fig. 4C). This could include the generation of a positive chemoattractant milieu by the enhanced expression of complement factor C3, the chemokine (C-C motif) ligands 2 and 3 and the local expression of proinflammatory...
cytokines, potentially boosting the immune response. Downregulation of the local expression of cyclooxygenase 2 (COX2), an enzyme required for producing immune-suppressive prostaglandins might contribute to an increase in local immune response.

The involvement of the adaptive immune system in O$_3$/O$_2$-PP–induced tumor defense is indicated by an enhanced expression of T-cell receptors and ligands involved in antigen presentation and induction of T-cell immune responses (Fig. 4D). The MHC class I–related protein CD1d, which specifically presents lipid antigens to T cells, was expressed at about 8-fold higher levels in remitting tumors. In addition, the T cell coreceptor CD4, necessary for MHC class II–dependent antigen detection, and the costimulatory binding couples CD80/CD28, CD86/CD28, and CD40/CD154 (CD40 ligand) were found elevated 2- to 6-fold (Fig. 4D). Thus, enhanced local antigen presentation seems to take place and might contribute to the tumoricidal immune response.

Differentiation of TILs into the T-cell subpopulations T1, T2, Tregs, and T17 according to the accepted classification, revealed a role of T1 and T2 cells in tumor regression (Fig. 4E). Expression of genes encoding receptors characteristic for T1 cells and IFNγ were significantly enhanced and the expression of the T2-associated cytokines IL4, IL5, and IL10 were induced only in animals with tumor remission (Fig. 4E). Although markers for Tregs (CCR8) and T17 cells (CCR6) were not significantly enhanced in tumors under remission, the transcription factor forkhead box P3 (FOXP3) was significantly elevated in tumors under remission, suggesting involvement of Tregs and/or T17 cells in tumor clearance. RAR-related orphan receptor C (RORC) expression was slightly reduced in remitting tumor tissues and IL17a was always absent (Fig. 4E).

Discussion

At the time of diagnosis, tumors have often developed to a macroscopically visible and clinically critical size. At this time, defense mechanisms of the body against tumor cells have failed, probably due to immune evasion of the tumor cells (17). In the worst case, tumor cells reprogram cells of the tumor microenvironment (TME), including immune cells to promote growth and vascularization of the tumor (18, 19). On the other hand, immune-based therapies have proved to be powerful weapons against cancer once the immune system is reprogrammed to detect the neoplastic cells. Novel ACT treatment paradigms showing that sensitization and activation of antitumorigenic leukocytes derived from the patient’s own immune system can be used as a therapeutic tool for cancer treatment (7, 20).

In this study, we were able to trigger such a tumoricidal immune response after application of a repetitive highly oxidative stimulus by insufflation of a medical O$_3$/O$_2$ gas mixture into the peritoneal cavity. O$_3$/O$_2$-PP treatment led to resistance against the tumor, which was transferable by ACT. In contrast with the ACT of ex vivo stimulated TILs, however, the O$_3$/O$_2$-PP approach does not require surgical preparation of tumor cells and TILs from solid tumors, which are often difficult to obtain.

We observed that O$_3$/O$_2$-PP treatment led to a significant increase of peripheral WBCs and CD3$^+$ TILs in the VX2 tumor tissue, compared with sham-treated tumors. If the increase in WBCs does rely on the O$_3$/O$_2$-PP treatment alone, or might reflect the beginning of an expansion of antitumorigenic leukocytes, is unknown. The increase of CD3$^+$ TILs is in accordance with clinical studies showing that high abundance of TILs in tumors is often associated with increased survival of patients with cancer (4, 21–23), whereas low density of intra- and peritumoral CD3$^+$, CD4$^+$, and CD8$^+$ cells is associated with increased risk of relapse as reported for squamous cell cervical cancer (24). Apparently, TILs are major players in cancer immune surveillance by virtue of their capacity to recognize, capture, and eliminate transformed cells (25, 26).

The molecular signature of immune response genes, measured in VX2 tumors remitting after O$_3$/O$_2$-PP treatment, indicates an enhanced activation of the innate and adaptive arms of the immune system, implicating a role of activated TILs.

The upregulation of the PRRs in remitting VX2 tumors may reflect an induction or increase in the sensitivity to detect ongoing pathologic changes and might be essential for triggering the tumoricidal response. Damage-associated molecular pattern molecules (DAMP refs. 27, 28) such as DNA/RNA fragments, glucose, or ATP released from VX2 tumor cells by itself or from cells of the TME might function as ligands for these PRRs (29). In fact, DAMPs are often critical for tumor development. For example, pathophysiologically released ATP was described as an activator of the NOD-like receptors (NLR) P3 inflammasome, which mediates the cleavage of pro-IL1β and pro-IL18 to biologically active cytokines or might subsequently trigger pyroptosis.
Two central components of the inflammasome [the platform protein NLR family, pyrin domain containing 3 (NLRC3) and the effector protein caspase 1; ref. 32] were upregulated in remitting VX2 tumors. This might imply the formation and activation of inflammasomes within the TME and might be responsible for the increase in IL18 gene expression (33).

The signature of enhanced PRR mRNAs might indicate an antitumorigenic effect of TLRs, NLRs, and RIG-I–like receptors in remitting VX2 tumors, albeit protumorigenic effects of PRRs are also known (34, 35).

Activation of PRRs leads to initiation of several signal transduction pathways resulting either in the synthesis of type I interferons (IFN), cytokines, and growth factors or in pyroptosis of the cell (36). Consistent with the observed expression of PRRs, an increase of numerous related downstream signaling molecules and transcription factors was noted in remitting VX2 tumors. These include the essential signal molecule myeloid differentiation primary response gene 88-like (MyD88), which, in relationship to TLR2, TLR4, TLR6, is known to result in mitogen-activated protein kinases and/or nuclear factor kappa-light-chain-enhancer of activated B cells 2 (NF-kB)–dependent expression of several inflammatory cytokines (36), especially the synthesis of pro-IL1β and pro-IL18. Surprisingly, NF-kB inhibitor alpha (NF-kBIA), an inhibitor of NF-kB is also enhanced in remitting VX2 tumors. However, enhanced NF-kBIA seemed unable to efficiently block NF-kB–dependent gene transcription of cytokines, because tumor necrosis factor alpha (TNFα), MIP1α, and interferon gamma (IFNγ) mRNAs were still detectable at significant levels in remitting VX2 tumors. This discrepancy might be explained by the expression of NF-kB and NF-kBIA in different cell populations within the tumor tissue.

Expression of COX2 was decreased in the VX2 tumors under O3/O2-PP–induced remission. As protumorigenic effects of COX2-derived prostaglandins such as PGE2 are related to tumor growth, angiogenesis, and inhibition of apoptosis (37), the diminished COX2 expression in remitting tumors is likely to result in a reduction of tumor permissive factors in the tumor microenvironment. In fact, blockade of COX2 by specific inhibitors is actually used for cancer chemotherapy (38, 39) and blockade of COX2 by the unspecific COX-inhibitor acetylsalicylic acid gains significance (40). Furthermore, factors involved in chemotractions namely complement factor C3, CCL2, and CCL3 might be causally involved in the increase of TILs in remitting VX2 tumors.

The presentation of tumor antigens in combination with costimulatory signals by antigen-presenting cells (APCs) is essential for utilization of the adaptive arm of the immune system. The observation of enhanced expression of the binding partners CD80/CD28, CD86/CD28, CD40/CD154 (CD40L), the T-helper cell specific CD4, and the MHC I–related receptor CD1d in remitting VX2 tumors supports our view of a decisive role of TILs in tumor defense. In this context, it is interesting to point out that the VX2 carcinoma initially developed after Shope papillomavirus–induced transformation of normal keratinocytes (12). Similarly, about 25% of HNSCC tumors or more, dependent on their anatomic site, are associated with high-risk human papillomaviruses (HPV), in particular the type 16 (41). The papillomavirus oncoprotein E5 is implicated in MHC class I downregulation (42, 43), and was shown to affect MHC class II maturation in IFNy-treated keratinocytes (44). Consistent with these reports, the VX2 carcinoma was found to express E5 mRNA at high levels (data not shown). Overexpression of E5, therefore, could be related to the observed immune escape of the VX2 tumor that was overcome by immune-modulatory effects of the O3/O2-PP treatment.

Further gene expression analysis of T-cell subtype–specific receptors and cytokines points to an involvement of effector T1 and T2 cells. The exact role of Tregs and T17 cells in the VX2 tumor model remains to be unraveled in studies to come. On the basis of the measured FOXP3 expression levels in remitting tumors, a functional involvement of these regulatory cell types seems likely, although other markers relevant for the identification of these cell types were not significantly enhanced. Both cell types were not significantly enhanced. Both cell types were not significantly enhanced.

Figure 4. Expression levels of immune-relevant genes in the tumor tissue. Immunologically relevant factors were grouped according to their mode of action in pattern recognition receptors (A), signal transduction factors (B), mediators or regulators of inflammatory responses (C), molecules functionally involved in antigen presentation to T cells and T-cell activation by essential costimulatory signals (D), and T-cell subpopulations identified by their specific expression pattern (E). Shown are mean values of fold changes in mRNA levels from solid tumors under remission (induced by O3/O2-PP, n = 6) compared with mRNA expression levels of tumors under progression (unaffected by sham treatment, n = 6). Values above 0.0 represent increased expression levels in the remitting tumor. Significant differences (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001) between tumors under regression and progression were calculated with the unpaired t test; n.s., not significant; n.d., not detected. If a given factor was induced in one experimental group only, a scatter plot of ΔCt values is depicted on the right side of the equivalent bar graphs. Gray areas in each scatter plot represent the range that was defined as negative (mRNA expression not verifiable due to a Ct value > 35 cycles = ΔCt < 0.00047). In this case, a nonparametric two-tailed Mann–Whitney test of the ΔCt values was used. Abbreviations in bars below the X-axes: TLR, Toll-like receptors; RLR, RIG-I–like receptors; trans.f., transcription factor; sup., immune suppressive; chem.at., chemotherapeutic; pro.inf., proinflammatory; AP, antigen presentation; T1, T2, T17; type 1, 2, 17 T cell. Abbreviations of genes: C3, complement component 3; CCL2, 3, chemokine (C-C motif) ligand 2, 3; CCR4, 5, 6, 8, chemokine (C-C motif) receptor 4, 5, 6, 7, 8; CD1d, 4, 28, 40, 86, 154, cluster of differentiation CD1d, 4, 28, 40, 86, 154; C-Jun, C-jun transcription factor; CXCR3, 4, chemokine (C-X-C motif) receptor 3, 4; IRAK1, interleukin 1 receptor–associated kinase 1-like; IRRF1, interferon regulatory factor 1; MAPK3, 1, 3, 8, 14, mitogen-activated protein kinase 1-like, 3-like, 8, 14; MDA-5, melanoma differentiation-associated gene 5; NOD1, 2, nucleotide-binding oligomerization domain containing 1, 2; RIG-1, retinoic acid inducible gene-I; STAT1, signal transducer and activator of transcription 1; TRAF6: TNF receptor–associated factor 6; TYK2: tyrosine kinase 2-like.
types, Tregs and TH17, have been described to exhibit pro-
as well as antitumorigenic effects in different types of
human cancers (reviewed in refs. 45, 46). The combined
upregulation of IL2, IL10, IL18, and IFN$$\gamma$$ suggests that an
immune response driven by TH1 and cytotoxic T cells and
NK cells might preponderate in remitting tumors. This is
in line with data, showing that IL10 in combination with
IL2 can increase the cytolytic activity of CD8$$^+$$ T cells (47),
and in combination with IL18 the cytolytic activity of NK
cells (48).

The most likely mechanism by which O3/O2-PP induces
the tumoricidal response seems to be related to the stim-
ulation of immune cells in consequence of the oxidative gas
insufflated into the peritoneum. This probably includes
antitumorigenic T cells but also resident macrophages and
dendritic cells (DCs). How the highly oxidative stimulus

Figure 5. Tumor resistance induced by ACT of PBLs. A, experimental design of the ACT. O3/O2-PP–treated rabbits that have been able to clear the tumor or
sham-treated, tumor sick rabbits, both derived from experimental phase I, were used as donor animals for PBL transfer. B, mean body temperature ± SEM of
recipient animals before and after ACT. The physiologic range of body temperature of NZW rabbits is marked in gray. C, VX2 tumor size development after
simultaneous VX2 tumor cell inoculation and ACT of PBLs derived from either O3/O2-PP–treated, cured, or sham-treated (tumor sick) animals. Shown are
the mean values ± SEM of the VX2 tumor size from all progressively growing tumors within the PBL-sham group and from remitting tumors of the PBL-O3/O2-
PP group. The dashed line represents the moving average over 3 days of progressively growing tumors of the PBL-O3/O2-PP group. D, Kaplan–Meier analysis
of the TTR probability and the TTP probability (E) of solid auricular VX2 tumors. Statistically significant (\( P < 0.05 \)) changes between the two groups were
calculated by the log-rank test; numbers and percentages of tumors that had reached the defined tumor volume characteristic for progression or regression
are shown in brackets. Mean values ± SEM are shown.
within the peritoneal cavity reaches and affects these leukocytes within the tumor tissue is at present unclear. In principle, two starting points of the gas are conceivable (Fig. 6). On the one hand, the radical gas could act directly on circulating immune cells (49) within the peritoneal cavity, and either activate antitumorigenic leukocytes, or destroy immune-suppressive immune cells, e.g., suppressor T cells, which until then had blocked antitumorigenic cells. On the other hand, oxidation of numerous biomolecules such as lipoproteins can lead to the generation of reactive oxygen species (ROS) and lipid oxidation products (LOP; ref. 50), which can function as DAMPs (51) within the peritoneum, or locally after reaching the tumor via the blood stream. In fact, ROS endogenously produced in case of cellular stress have been found to function as DAMPs, and are thought to be common initiators of the NLRP3 inflammasome pathway (33, 51, 52).

Unequivocal proof that immune cells are responsible for VX2 tumor eradication has been made by adoptive transfer of isolated PBLs. The ACT of PBLs derived from O3/O2-PP–treated animals, which had been able to clear the primary tumor, also led to tumor clearance in host animals with a 60% success rate. We suggest that the adaptive immune system is crucially involved in the O3/O2-PP–induced tumor defense and that long lasting “memory” cells against the VX2 tumor must have developed. Thus, medically implemented oxidative stress by O3/O2-PP is suggested as a novel therapeutic approach to induce sustainable adoptively transferable oncolytic immune responses.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A. Rossmann, R. Mandic, H. Höflken, E. Weihe, M. Bette
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