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Original Paper

Hypoxia Enhances Immunosuppression by Inhibiting CD4+ Effector T Cell Function and Promoting Treg Activity

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Key Words
Colitis-associated colon cancer • Hypoxia • HIF-1a • T cell function

Abstract
Background/Aims: Hypoxia occurs in many pathological conditions, including inflammation and cancer. Within this context, hypoxia was shown to inhibit but also to promote T cell responses. Due to this controversial function, we aimed to explore whether an insufficient anti-tumour response during colitis-associated colon cancer could be ascribed to a hypoxic microenvironment. ***Methods:*** Colitis-associated colon cancer was induced in wildtype mice, and hypoxia as well as T cell immunity were analysed in the colonic tumour tissues. In addition, $CD4^+$ effector T cells and regulatory T cells were cultured under normoxic and hypoxic conditions and examined regarding their phenotype and function. ***Results:*** We observed severe hypoxia in the colon of mice suffering from colitis-associated colon cancer that was accompanied by a reduced differentiation of $CD4^+$ effector T cells and an enhanced number and suppressive activity of regulatory T cells. Complementary *ex vivo* and *in vitro* studies revealed that T cell stimulation under hypoxic conditions inhibited the differentiation, proliferation and IFN-γ production of T_H1 cells and enhanced the suppressive capacity of regulatory T cells. Moreover, we identified an active role for HIF-1α in the modulation of $CD4^+$ T cell functions under hypoxic conditions. ***Conclusion:*** Our data indicate that oxygen availability can function as a local modulator of $CD4^+$ T cell responses and thus influences tumour immune surveillance in inflammation-associated colon cancer.

Introduction

Colorectal cancer is a major cause of cancer-related death in many countries. In recent years, new hypotheses on the mechanisms involved in colorectal carcinogenesis have been considered. It has been postulated that colorectal injury induced by inflammation, as it is seen in patients with ulcerative colitis, is strongly associated with the development of colon cancer

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[1]. The excessive inflammation is initiated by innate immune cells, but further pathology is driven by a prevalent activation of T helper cells [2]. If the immune response fails to resolve the injury, a tumour-prone microenvironment with prolonged uncontrolled proliferation in attempt to repair the damage may result. Epithelial oxygen tension plays a critical role in the resolution of inflammation [3]. Under homeostasis, the gastrointestinal tract has been described to subsist in a state of physiologically low oxygen level. More precisely, intestinal epithelial cells have a steep oxygen gradient where the tips on the villi are hypoxic and the oxygenation increases at the base of the villi. Under intestinal inflammation, there is a dramatic shift in tissue metabolism and perfusion, which results in heightening of hypoxia throughout the mucosa. In the inflamed mucosa, hypoxia increases barrier protective genes, elicits protective innate immune response, and activates an antimicrobial response by the induction of β-defensins [3]. On the other hand, the presence of intratumoural hypoxia has been identified as a negative prognostic and predictive factor in various human solid tumours and this has been attributed to its multiple contributions to chemoresistance, radioresistance, angiogenesis, resistance to cell death, altered metabolism, and genomic instability [4-7].

In addition, the hypoxic tumour microenvironment profoundly influences the host immune response. It is well established that T cells in the context of established progressing tumours exhibit an anergic or exhausted state leading to T cell-intrinsic dysfunction [8]. Furthermore, a variety of solid tumours possess an increased amount of immunosuppressive cells, such as regulatory T cells (Tregs) and immunosuppressive cytokines derived from the Tregs or the tumour cells [9, 10]. Increasing evidence now suggests that oxygen availability can regulate T cell differentiation and function, e.g. human T cells activated under hypoxia are protected from activation-induced cell death [11]. Conversely, cultivating activated human T cells at physiologic O_2 can result in a significantly elevated rate of spontaneous apoptosis in certain T cell subsets [12]. Furthermore, it was demonstrated that *in vitro* oxygen availability also influences cytokine production [13].

Due to the strong impact of hypoxia on immune responses during inflammation and cancer, we combined both issues and examined whether the impaired colonic $CD4^+$ T cell response during colitis-associated colon cancer (CAC) could be associated with a hypoxic microenvironment.

Materials and Methods

Mice

All animals used in this study were 8- to 12-week-old female and male mice bred and housed under specific pathogen-free conditions in the Laboratory Animal Facility of the University Hospital Essen. BALB/c mice were obtained from Harlan Winkelmann GmbH (Borchen, Germany). Foxp3/eGFP reporter mice, which express both Foxp3 and green fluorescent protein (GFP) under the endogenous regulatory sequence of the Foxp3 locus, were obtained from the Jackson Laboratory (USA).

AOM/DSS protocol

Colitis-associated colon cancer was induced in BALB/c mice as previously described [14]. Mice were injected intraperitoneally (ip) with the procarcinogen azoxymethane (AOM; 12.5 mg per kg of body weight) (Sigma-Aldrich, St. Louis, MO), followed by 3 cycles of 3% dextran sulphate sodium salt (DSS, MP Biomedicals, Eschwege, Germany; MW, 36–50 kDa) given via the drinking water for 5 to 7 days. Mice were sacrificed at week 12.

Histology and immunohistochemistry of colon tissues

Tissue sections (6 µm) were prepared from paraffin-embedded tissue blocks and stained with hematoxylin and eosin (H&E). To assess colonic hypoxia, mice were treated intraperitoneally with pimonidazole hydrochloride (60 mg per kg of body weight) using the Hypoxyprobe™-1Kit (Hypoxyprobe Inc., Burlington, MA) 1 h prior to sacrifice. Visualization of hypoxia in colonic mucosa was then performed

by anti-pimonidazole staining and counterstaining with Meyers Hemalaun. In brief, tissue slides were deparaffinised, rehydrated and steam boiled in citrate buffer pH 6. Sections were sequentially blocked for 40 min with 5% BSA in TBS. Blocked sections were incubated with anti-pimonidazole mouse IgG1 monoclonal antibody (MAb1) in 5% BSA/ TBS over night at 4°C. Hypoxia was detected using the DAKO Real Detection System APAAP mouse K5000 (Dako Deutschland GmbH, Hamburg, Germany). Stained sections were counterstained with Meyers Hemalaun, rinsed in running tap water, and covered with Mowiol®.

To stain hypoxia by immunofluorescence tissue sections (6 µm) were prepared from colons frozen in liquid nitrogen. After thawing tissue sections were incubated for 10 min in sodium citrate buffer at 98°C for antigen retrieval. After washing with deionized water and PBS sections were blocked for 30 min with 2% normal goat serum in PBS supplemented with 0,01% Triton X-100. Mouse monoclonal anti-pimonidazole antibody Dylight™549 (clone 4.3.11.3, Hypoxyprobe Inc.) was diluted 1:200 in blocking solution. Tissue sections were incubated overnight at 4°C with the anti-pimonidazole antibody, washed with PBS, stained with Hoechst (1:1000, 2 min), and mounted in Fluoromount-G (Southern Biotech, Birmingham, AL). Immunofluorescence was analysed with the Axio Observer.Z1 (Zeiss, Jena, Germany) at 20x magnification using an ApoTome and AxioVision software (both Zeiss).

Isolation of lamina propria lymphocytes from the colon

Lamina propria lymphocytes were isolated as described previously [15], with minor modifications. Colons were flushed with PBS to remove faeces, opened longitudinally and cut into 1 cm pieces. Tissue pieces were washed twice in PBS containing 3 mM EDTA for 10 min at 37°C with rotation. EDTA was removed by washing colon pieces twice in RPMI containing 1% FCS, 1 mM EGTA, and 1.5 mM $MgCl_2$ for 15 min at 37°C with rotation. Colon pieces were then vortexed intensely, washed with PBS, and digested in RPMI containing 20% FCS and 100 U/mL collagenase (*Clostridium histolyticum,* Sigma-Aldrich, St. Louis, MO) at 37°C for 60 min. The remaining tissue was separated from cells by passing the cell suspension through a 40 µm cell strainer and washing with culture medium.

Antibodies and flow cytometry

T cells were stained with fluorochrome-labeled anti-mouse CD4 antibody (RM4-5, BD Biosciences, Heidelberg, Germany). For analysis of intracellular IFN-γ cells were stimulated for 4 h with 10 ng/mL PMA and 1 µg/mL ionomycin in the presence of 5 µg/mL Brefeldin A (all Sigma-Aldrich). After staining with anti-CD4 antibodies, cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% NP-40, and stained for intracellular IFN-γ with anti-mouse IFN-γ (XMG1.2, BD Biosciences). Intracellular detection of Foxp3 was performed with anti-Foxp3 (FJK-16s) using the Foxp3 staining kit from eBioscience (Frankfurt, Germany) according to the manufacturer's recommendations. Fixable Viablitity Dye (ebioscience) was used to stain for dead cells. Cells were analysed by flow cytometry on a LSR II instrument using DIVA software (both from BD Biosciences).

Proliferation assay

$CD4^+Foxp3^-$ T cells were sorted from the spleen of Foxp3/eGFP reporter mice using a FACSAria II cell sorter (BD Biosciences). CFSE labelled $CD4^+Foxp3^-$ T cells (4×10^5) were activated *in vitro* with plate-bound αCD3 (0.75 µg/ml) and soluble αCD28 (1 µg/ml) (both BD Biosciences) under normoxic (20-21% O_2) conditions or in a hypoxia chamber ($Invivo_2$ 400 Hypoxia Workstation, Ruskinn Technology Ltd., Bridgend, Great Britain) with low oxygen level (1% O_2, hypoxia) for 3 days. Every day, the total viable cell number was determined by trypan blue staining. The proliferation was assessed by the loss of fluorescent dye CFSE and measured on a LSR II instrument using DIVA software (both from BD Biosciences).

Suppression assay

$CD4^+Foxp3^+$ Tregs were separated from the colons of healthy mice and mice with colitis-associated colon cancer (CAC) (both Foxp3/eGFP reporter mice) using a FACSAria II cell sorter (BD Biosciences). $CD4^+$ T cells were purified from spleens of naïve mice with the $CD4^+$ T-cell isolation kit II (Miltenyi Biotec, Bergisch-Gladbach, Germany) and labelled with eFluor670 (eBioscience). $CD4^+$ responder T cells (1×10^5) were either cultured alone or co-cultured with $CD4^+Foxp3^+$ ($eGFP^+$) Tregs (1×10^5) for 3 days in the presence of 1 µg/mL αCD3 (2C11; BD Biosciences). Irradiated splenocytes from naïve BALB/c mice served as antigen-presenting cells (APCs) (3×10^5).

To analyse the suppressive capacity of Tregs under hypoxia, $CD4^+Foxp3^+$ Tregs and $CD4^+$ responder cells were isolated from the spleen of Foxp3/eGFP reporter mice. eFluor670 labelled $CD4^+$ responder T cells (2×10^5) were either cultured alone or co-cultured with $CD4^+Foxp3^+$ Tregs (2×10^5) for 3 days in the presence of 1 µg/mL αCD3 (2C11; BD Biosciences) under normoxic or hypoxic conditions. Proliferation of responder T cells was assessed by the loss of the fluorescent dye eFluor670 and measured on a LSR II instrument using DIVA software (both from BD Biosciences).

T_H1 differentiation assay

$CD4^+Foxp3^-$ T cell were sorted from the spleen of Foxp3/eGFP reporter mice using a FACSAria II cell sorter (BD Biosciences) and were activated *in vitro* with plate-bound αCD3 (0.75µg/ml) and soluble αCD28 (1 µg/ml) (both BD Biosciences) in the presence of recombinant mouse IL-12 (4 ng/ml) (R&D Systems, Wiesbaden, Germany) under normoxic or hypoxic conditions. At day 4, T_H1-cell-polarization was measured by FACS staining of the cells for IFN-γ.

Transcriptome analyses

Total RNA from $CD4^+$ T cells was isolated with the RNeasy kit (Qiagen, Hilden, Germany). The quality and integrity of the total RNA were controlled with an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Next, 500 ng of total RNA was used for a Cy3-labelling reaction with the one-color Quick Amp Labeling Kit (Agilent Technologies). Labeled cRNA was hybridized to Agilent's mouse 4x44k microarrays for 16 h at 68°C that were then scanned with the Agilent DNA Microarray Scanner. Expression values were calculated with the Feature Extraction 10.5.1.1 software package (Agilent Technologies).

Quantitative RT-PCR

RNA was isolated from sorted T cells using the RNeasy Kit (Qiagen) according to the manufacturer's instructions. RNA was obtained from colon biopsies using the RNeasy Fibrous Tissue Kit (Qiagen). Following DNase-mediated digestion (Qiagen), cDNA was synthesized with M-MLV Reverse Transcriptase (Promega, Mannheim, Germany) and OligodT mixed with Random Hexamer primers (Invitrogen, Karlsruhe, Germany) on an ABI PRISM cycler (Applied Biosystems, Life Technologies, Darmstadt, Germany). Real-time RT-PCR was performed using SYBR Green PCR kit and specific primers for IL-10, HIF-1α, BNIP3, GLUT1, GAPDH, VEGF, and RPS9. Relative RNA levels were determined with standard curves included for each individual gene and further normalization to the housekeeping gene RPS9.

Statistics

All results were expressed as means ± SEM. Differences were assessed by Student's t-test. Data analysis was performed with Prism 5.0 software (GraphPad, La Jolla, CA). Statistical significance was set at the level of $P < 0.05$.

Results and Discussion

Colitis-associated colon cancer induces a strong hypoxic and immunosuppressive microenvironment

To assess whether hypoxia is induced during CAC and whether low oxygen levels may impair an anti-tumour response, BALB/c mice were subjected to the azoxymethane (AOM)/dextran sulfate sodium (DSS) regimen, a well-established protocol for the induction of CAC [14, 16]. After injection of the procarcinogen AOM, the repeated administration of DSS via the drinking water induces a strong inflammatory response in the intestine. Mice lose as much as 10% of their initial body weight during the DSS cycles [16]. At week 12 after AOM/DSS treatment, we documented tumour development by endoscopy and histology (Fig. 1A). To determine the presence of hypoxia within the colonic tumour tissues, the binding of pimonidazole to macromolecules, which occurs in the absence of adequate oxygen levels, was evaluated. Consistent with earlier reports, physiologic hypoxia was observed in healthy animals on the colonic surface epithelium but not in the crypts (HC, Fig. 1B) [17]. In contrast,

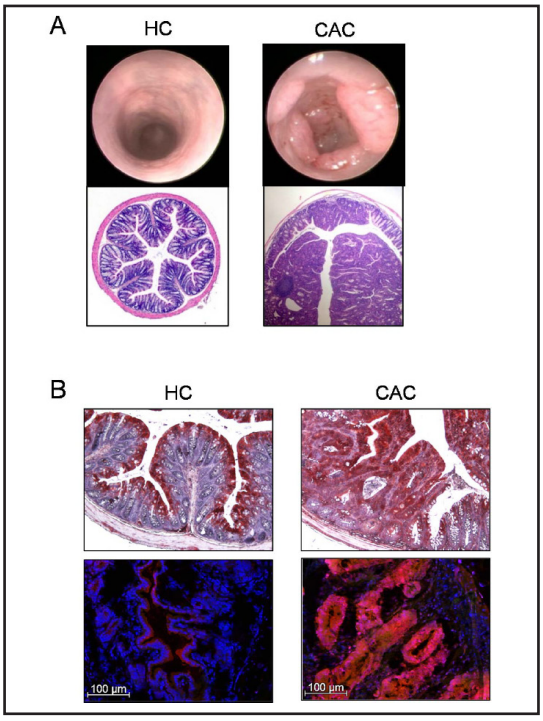


Fig. 1. Colitis-associated colon cancer is associated with severe hypoxia in the colon. To induce colitis-associated colon cancer, mice were given an intraperitoneal injection of the procarcinogen AOM followed by three cycles of DSS in the drinking water. (A) Representative endoscopic and histological images from the distal colon of healthy control mice (HC) and AOM/DSS-treated mice (CAC) at week 12. (B) To assess colonic hypoxia during colitis-associated colon cancer, mice were treated intraperitoneally with pimonidazole 1 h prior to sacrifice. Visualization of hypoxia in colonic mucosa was then performed by α-pimonidazole staining. Representative histological images from the distal colon of healthy control mice (HC) and AOM/DSS-treated mice (CAC) are shown (immunohistochemistry – upper panel; immunofluorescence – lower panel).

in week 12 after the initial induction of colitis-associated colon cancer, an increased colonic epithelial pimonidazole-uptake, extending to additional parts of the crypts, the submucosal layer and the tumour tissue, respectively, was observed in mice suffering from colitis-associated colon cancer (CAC, Fig. 1B).

Next, we compared the distribution of $CD4^+$ T cells in the colon of healthy mice and mice with CAC. In tumour-bearing colons, the proportion of $CD4^+$ T cells was significantly higher compared to the colon of healthy controls (Fig. 2A). However, when we analysed the effector activity of $CD4^+$ T cells by the determination of their cytokine production, we observed a significant reduction of $CD4^+$ T cells with the ability to produce INF-γ after restimulation *ex vivo* in the colon of CAC mice compared to healthy controls (Fig. 2B). This is well in line with the human situation, as decreased percentages of T_H1 cells were found in patients with colorectal tumours [18]. However, when we determined the absolute numbers of $CD4^+IFN\text{-}\gamma^+$ T cells in the colons, we identified an increase in these cells in mice suffering from CAC, probably due to the significant increase in the percentage of $CD4^+$ T cells. This implies that hypoxia is influencing the differentiation of effector T cells rather than their ability to produce IFN-γ.

Besides the anergic or exhausted state of effector T cells in tumours, a variety of solid tumours reveal an increase in immunosuppressive cells, such as Tregs [19, 20]. Importantly, a strong accumulation of $Foxp3^+$ regulatory $CD4^+$ T cells was detected in the colons of mice suffering from CAC but not in the colon of healthy control mice (Fig. 2C). To further explore the suppressive activity of Tregs in the hypoxic colonic tumour tissue, we sorted $CD4^+Foxp3^+$ Tregs from the colons of healthy and CAC-diseased mice and co-cultured them with $CD4^+$ responder T cells. As demonstrated in Fig. 2D, we observed that the ability of $CD4^+Foxp3^+$ Tregs to suppress the proliferation of responder $CD4^+$ T cells was enhanced when these cells were isolated from CAC mice. This is well in line with recent studies demonstrating that Tregs isolated from human and mouse colonic tumour tissues strongly suppressed the proliferation of tumour-infiltrating $CD8^+$ and $CD4^+$ T cells [21, 22].

IL-10 is particularly important for the function of Tregs at environmental interfaces at which tolerance induction is the hallmark of intestinal homeostasis [23, 24]. To get a hint whether IL-10 may exert the inhibitory mechanism during CAC, we performed Luminex analysis of secreted IL-10 from colon biopsies of healthy and CAC mice. Indeed, IL-10

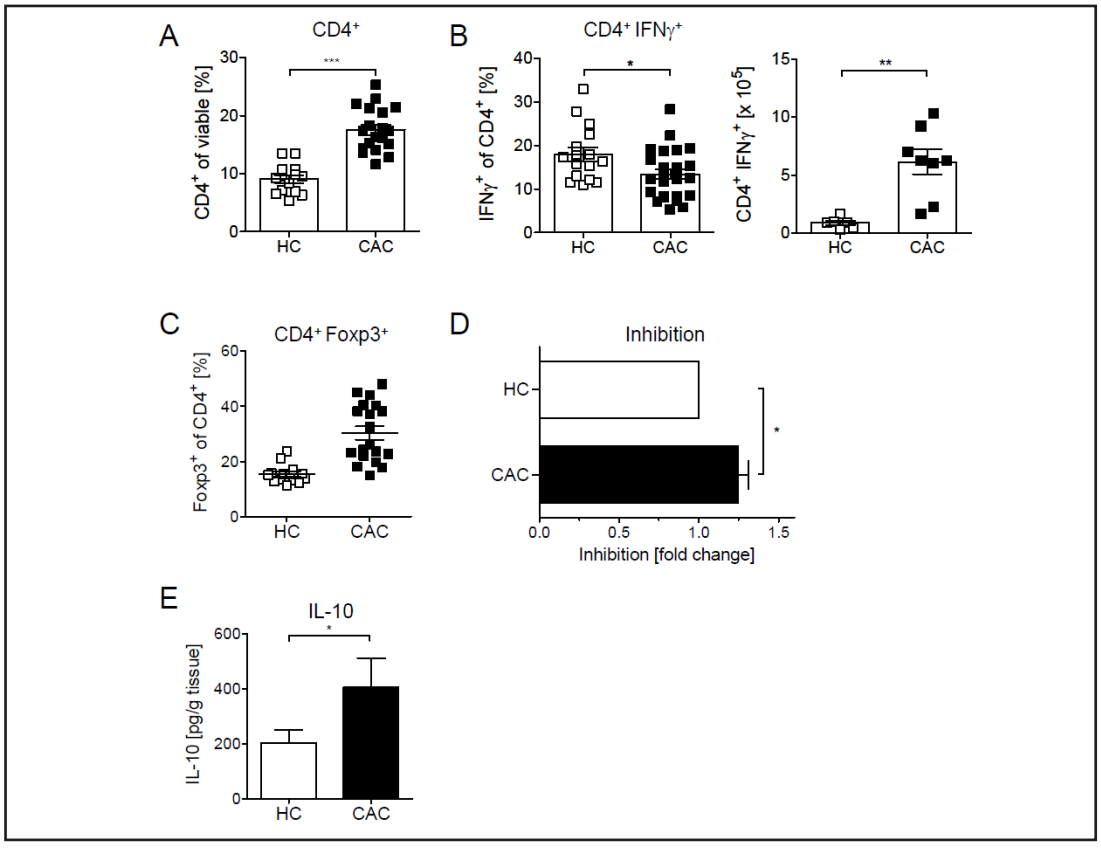


Fig. 2. Colitis-associated colon cancer induces a strong immunosuppressive microenvironment in the colon. Colitis-associated colon cancer (CAC) was induced in BALB/c or Foxp3/eGFP reporter mice. (A-C) At week 12, LPLs from the colon were isolated and stained with antibodies against CD4, IFN-γ, Foxp3 and viability dye, and analysed by flow cytometry. (A) Percentages of $CD4^+$ T cells on gated viable cells are demonstrated. (B) LPLs were cultured for 4 h in the presence of PMA, ionomycin and brefeldin A, and percentage of IFN-γ on gated viable $CD4^+$ T cells was determined. Absolute numbers of $CD4^+IFN\text{-}\gamma^+$ per colon was calculated. (C) Percentage of Foxp3 among gated viable $CD4^+$ T cells is shown. Scatter plots indicate the mean value with the standard error of the mean (SEM). The significance of the differences between healthy control mice (HC) and CAC mice was calculated with unpaired Student's t test (* $p < 0.05$; *** $p < 0.001$). (D) $CD4^+eGFP^+$ ($Foxp3^+$) T cells (Treg) from colons of AOM/DSS-treated (CAC) or healthy (HC) Foxp3/eGFP reporter mice were sort-purified. Treg were cultured at a 1:1 ratio with eFluor-labelled $CD4^+$ responder T cells with antigen-presenting cells in the presence of αCD3. Proliferation of responder T cells was measured by the loss of eFluor dye and inhibition was calculated. Inhibition of healthy control Tregs was set to 1 and the values of CAC Tregs were adjusted to them. Bar diagrams represent the inhibition as mean ± SEM of three independent experiments. Statistical analysis was performed with paired Student's t-test (*, $p < 0.05$). (E) At week 12 after CAC induction a biopsy from the colon was incubated for 6 hours in culture medium. IL-10 concentrations in the supernatants were determined. Bars show the mean of the amount of IL-10 per gram of tissue ± SEM from HC n = 10 and CAC n = 10 individual mice.

secretion was increased in the tumorous tissue (Fig. 2E). Collectively, we observed severe hypoxia in the colon of mice suffering from CAC that was accompanied by a reduced $CD4^+$ effector T cell differentiation and an enhanced number and suppressive activity of Tregs.

Hypoxia down-regulates the proliferation and the effector function of $CD4^+$ T cells

In order to analyse in more detail, whether hypoxia directly alters $CD4^+$ T cell functions, $CD4^+Foxp3^-$ T cells were purified from the spleen of mice, CFSE labelled, and stimulated with αCD3/αCD28 for three days under either hypoxic or normoxic conditions. As depicted in Fig. 3A, the cell numbers of unstimulated $CD4^+Foxp3^-$ T cells stayed constant over the course of

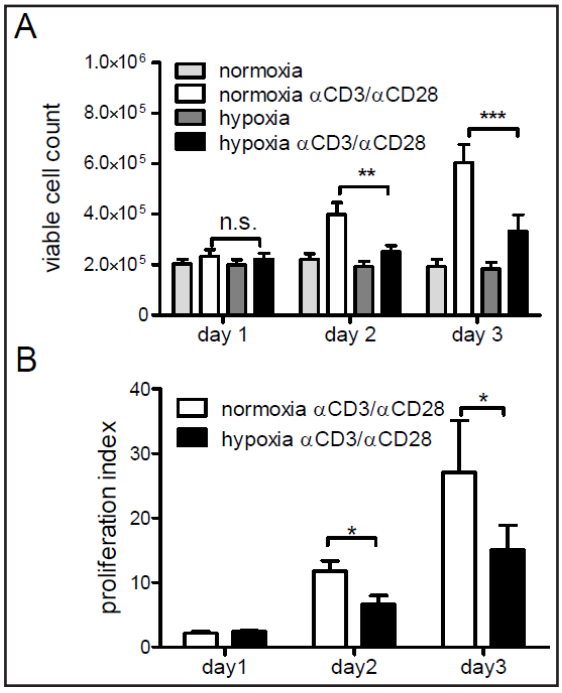


Fig. 3. Hypoxia inhibits T cell proliferation. CFSE labelled CD4⁺Fopx3⁻ T cells were stimulated with αCD3/αCD28 for 3 days under hypoxic or normoxic conditions. (A) Every day, the viable cell number was determined by trypan blue staining. Bar diagrams represent the number of viable cells as mean ± SEM of 3 independent experiments. Statistical analysis was performed with paired Student's t-test (** $p < 0.01$; *** $p < 0.001$; n.s. not significant). (B) At indicated time points, cells were analysed by flow cytometry for the loss of CFSE dye. The proliferation index was calculated by setting the mean fluorescent intensity values of the unstimulated cells to 100% and adjusting the values of the corresponding stimulated cells accordingly. The reciprocal value is displayed as the proliferation index. All experiments were performed in duplicates and the mean value of three independent experiments is shown. Bar diagrams represent the proliferation as mean ± SEM of three independent experiments. Statistical analysis was performed with paired Student's t-test (* $p<0.05$).

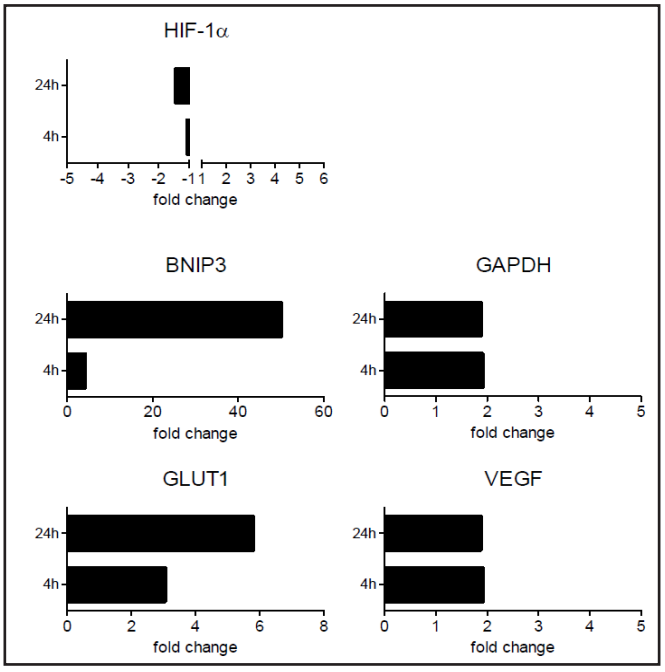


Fig. 4. Expression of HIF-1α and selected hypoxia-inducible genes in effector T cells. CD4⁺Foxp3⁻ T cells were stimulated with αCD3/αCD28 under either hypoxic or normoxic conditions for 4 h or 24 h respectively. RNA was isolated at indicated time points, transcribed into cDNA and used for quantitative real-time PCR. The expression was normalized to the housekeeping gene RPS9. The fold changes of the mean quantity of hypoxic versus normoxic samples are displayed.

the experiment, both under normoxic and hypoxic conditions. In contrast, the cell numbers of stimulated T cells increased steadily, but to a significantly higher extent under normoxic than under hypoxic conditions (Fig. 3A). In addition, the proliferation of CD4⁺ T cells was examined. After 24 h, no significant difference in the proliferation rate was detectable. Interestingly, from the second day onwards the proliferation of CD4⁺ T cells stimulated under normoxia was significantly higher than the proliferation of CD4⁺ T cells stimulated under hypoxic conditions (Fig. 3B). In summary, both, viable cell counts and proliferation analyses indicate that hypoxia inhibits the proliferation of CD4⁺ T cells *in vitro*. These results are in parts consistent with a former study, which demonstrates that the proliferation of human PBMCs is diminished under hypoxia due to elevated apoptosis [12].

Table 1. Transcriptome analysis of selected genes in effector CD4⁺ T cells

Common Name	GenBank Accession Number	Signal Intensity		Fold change Hypoxia *versus* Normoxia
		Normoxia	Hypoxia	
IL-2	NM_008366	5246.44	4316.18	-1.22
IL-9	NM_008373	164.53	22.06	-7.46
IL-10	NM_010548	103.63	189.06	1.82
IL-17f	NM_145856	248.48	75.33	-3.30
IFN-γ	NM_008337	17659.69	6718.72	-2.63
HIF-1α	NM_010431	3766.47	2736.74	-1.39
BNIP3	NM_009760	214.97	1027.90	4.79
GAPDH	NM_008084	17185.15	27899.93	1.62
GLUT1	NM_011400	3693.79	9192.70	2.49
VEGF	NM_009505	207.02	238.04	1.15

To get a comprehensive overview about the effector function of T cells under hypoxia, transcriptome analyses of CD4⁺ effector T cells, stimulated under hypoxic or normoxic conditions, were performed. Interestingly, the production of classical pro-inflammatory cytokines, such as IL-2, IL-17 and IFN-γ, was reduced in CD4⁺ T cells stimulated under hypoxic conditions compared to CD4⁺ T cells stimulated under normoxic conditions (Table 1). Of note, the strongest downregulation of mRNA expression levels was detected for IL-9. IL-9 is critical in controlling immune-associated diseases, and T_H9 cells have been reported to inhibit tumour growth [25, 26]. However, further studies are needed to clarify in detail how hypoxia interferes with the generation of T_H9 cells.

In contrast to the downregulation of pro-inflammatory gene expression, the expression of the anti-inflammatory cytokine IL-10 was upregulated under hypoxia. Since IL-10 was also elevated during CAC, these findings suggest that tumour hypoxia may contribute to the induction of an immunosuppressive microenvironment. Interestingly, Shehade et al. proposed that the reduced expression of IFN-γ under hypoxia is a direct effect of enhanced IL-10 production [27].

HIF-1α is an important transcription factor that is only activated under hypoxic conditions [28]. Consequently, we examined whether the impact of hypoxia on CD4⁺ effector T cell function could be due to the stabilization of HIF-1α. Therefore, we analysed the transcriptome data regarding the expression of HIF-1α and HIF-1α target genes. Nearly no differences in the expression of HIF-1α were detected between CD4⁺ T cells stimulated under normoxic or hypoxic conditions. In contrast, hypoxia-inducible genes, such as BNIP3 (BCL2/adenovirus E1B 19 kDa protein-interacting protein 3) and GLUT1 (glucose transporter 1), which are known to be regulated by HIF-1α, were moderately upregulated after 4 h exposure to hypoxia. The expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and VEGF (vascular endothelial growth factor), two further target genes of HIF-1α, was only slightly increased under hypoxia (Table 1).

To validate gene expression data by a second method, naïve CD4⁺Foxp3⁻ T cells were stimulated under either normoxic or hypoxic conditions for 4 h and 24 h respectively, and quantitative real-time PCR was performed for HIF-1α, BNIP3, GAPDH, GLUT1 and VEGF. The expression of HIF-1α was again not altered during hypoxia. In contrast, BNIP3 and GLUT1 were strongly upregulated after 4 h and 24 h (Fig. 4) and VEGF and GAPDH displayed only minimal changes in expression which is in line with the transcriptome analyses. Interestingly, it was shown before that T cells lacking HIF-1α produce higher levels of pro-inflammatory cytokines and that this effect can be at least partially explained by a significantly increased NF-kB activation in TCR activated HIF-1α-deficient T cells [29]. Together, these results propose an active role for HIF-1α in the modulation of CD4⁺ effector T cell function under hypoxic conditions.

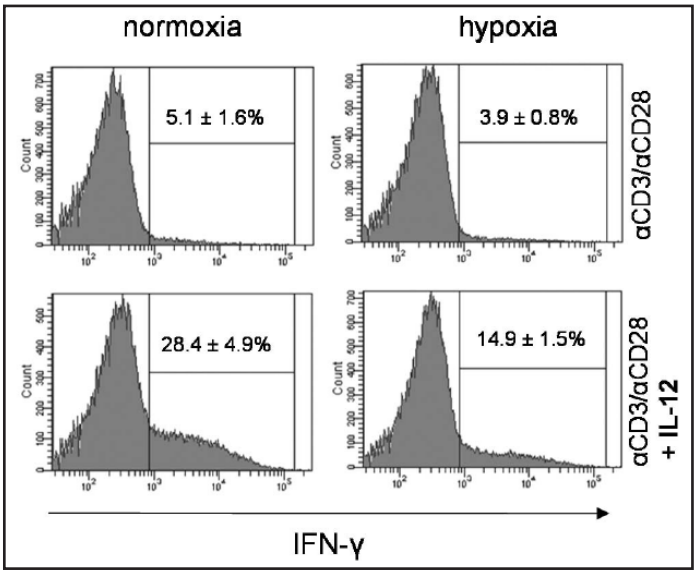


Fig. 5. The differentiation of naïve T cells into T_H1 cells is impaired under hypoxia. $CD4^+$ T cells were stimulated with αCD3/αCD28 in the presence of IL-12 to induce T_H1 differentiation either under normoxic or hypoxic conditions. After 4 days, the expression of IFN-γ was analysed by flow cytometry. The histograms show the expression of INF-$γ^+$ among $CD4^+$ T cells. One representative experiment of three independent experiments is displayed. Percentages indicate the mean percentage of IFN-$γ^+$ T cells and the corresponding standard error of the mean.

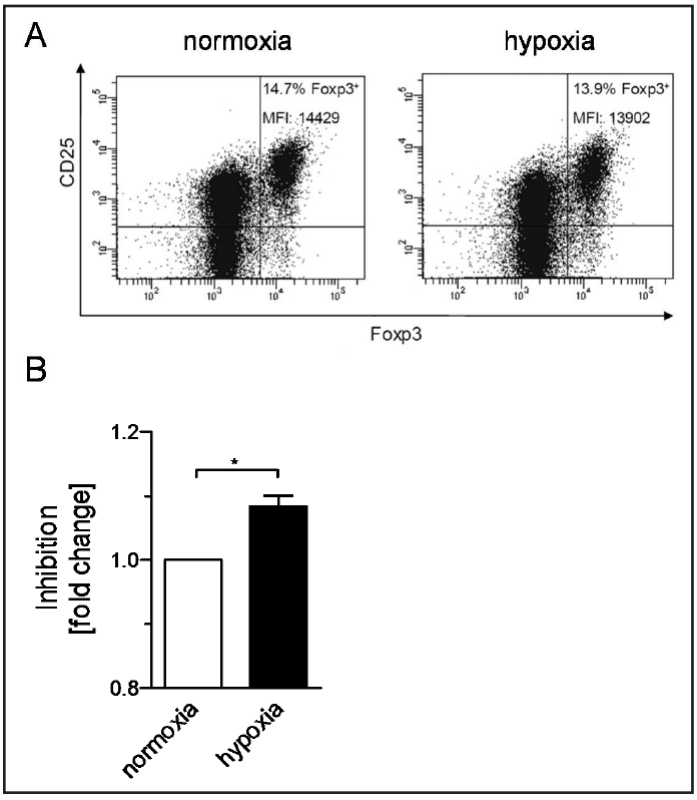


Fig. 6. Hypoxia does not alter the Foxp3 expression in $CD4^+$ Tregs but increases the inhibitory capacity. (A) Total $CD4^+$ T cells were stimulated with αCD3/αCD28 under either hypoxic or normoxic conditions for 4 h or 24 h. The cells were harvested and the expression of Foxp3 in $CD4^+CD25^+$ T cells was analysed by flow cytometry. The percentage of $Foxp3^+$ T cells and the mean fluorescent intensity (MFI) are indicated. One representative experiment out of three independent experiments is displayed. (B) $CD4^+$ $Foxp3^+$ Tregs were co-cultured at a ratio of 1:1 with eFluor-labelled $CD4^+$ responder T cells in the presence or absence of αCD3 under normoxic or hypoxic conditions. Proliferation of responder T cells was measured by the loss of eFluor dye, and inhibition was calculated. Inhibition of Tregs cultivated in normoxia was set to 1 and the values of Tregs cultivated in hypoxia were adjusted to them. Bar diagrams represent the inhibition as mean ± SEM of three independent experiments. Statistical analysis was performed with paired Student's t-test (* $p < 0.05$).

Hypoxia inhibits the differentiation of T_H1 cells

IFN-γ is an important player for an effective anti-tumour response [30], and the expression was altered in the colon of mice suffering from CAC, and under hypoxic conditions *in vitro*. Therefore, we measured whether hypoxia not only influences the T_H1 effector function but also the differentiation of naïve $CD4^+$ T cells into T_H1 effector cells. For this, $CD4^+$ T cells were stimulated in the presence of IL-12 under normoxic and hypoxic conditions. At day 4, intracellular cytokine staining was performed to determine the percentage of IFN-

Table 2. Transcriptome analysis of selected genes in CD4⁺ Tregs

Common Name	GenBank Accession Number	Signal Intensity		Fold change Hypoxia *versus* Normoxia
		Normoxia	Hypoxia	
IL-2	NM_008366	147.65	143.13	-1.03
IL-9	NM_008373	161.18	24.39	-6.61
IL-10	NM_010548	444.32	606.09	1.37
IL-17f	NM_145856	36.21	24.00	-1.51
IFN-γ	NM_008337	1889.75	977.13	-1.93
HIF-1α	NM_010431	5226.57	4984.58	-1.05
BNIP3	NM_009760	420.36	4208.08	10.01
GAPDH	NM_008084	28471.80	59179.42	2.08
GLUT1	NM_011400	4678.63	20609.31	4.40
VEGF	NM_009505	63.12	132.63	2.10

γ-producing T_H1 cells. Our data indicate that the proportion of IFN-γ-producing T_H1 cells was strongly decreased under hypoxic compared to normoxic conditions (Fig. 5). Recent studies suggested that the enhanced expression of IL-10 during hypoxia directly inhibits T_H1 priming [31-33], and that STAT3 activation under hypoxia decreases T_H1 activation [27, 34]. However, further investigations are required to identify the exact mechanisms.

Hypoxia modulates the function of regulatory T cells

As we have identified an enhanced percentage of Tregs in the hypoxic colon of mice suffering from CAC, we next studied whether hypoxia influences the phenotype and function of Tregs. We stimulated CD4⁺ T cell under normoxic and hypoxic conditions and analysed the expression of Foxp3, which is the transcription factor for Tregs [35], by flow cytometry. As demonstrated in Fig. 6A, the expression of Foxp3 was neither modulated after 4 h, nor after 24 h of hypoxia (data not shown). Similar results were obtained in a recent study demonstrating that hypoxia does not enhance the Treg abundance *in vitro* [27].

To determine whether hypoxia alters the function of Tregs, transcriptome analyses of CD4⁺Foxp3⁺ Tregs, stimulated under normoxic and hypoxic conditions, were performed in accordance to the analyses of CD4⁺ effector T cells. As already detected for CD4⁺ effector T cells, the rather low basal expression of pro-inflammatory cytokines by Tregs was further downregulated, whereas the expression of IL-10 was upregulated under hypoxic compared to normoxic conditions (Table 2). This is not surprising as Tregs are not considered as sources of inflammatory cytokines. To assess whether the altered cytokine production in hypoxia may elevate the inhibitory activity of Tregs, we isolated CD4⁺Foxp3⁺ Tregs from the spleen of mice and co-cultured them with CD4⁺ responder T cells under normoxic and hypoxic conditions for 3 days. The inhibitory effect of Tregs was slightly, but significantly increased under hypoxic compared to normoxic conditions (Fig. 6B).

High surface expression of programmed cell death 1 (PD-1) is associated with T-cell exhaustion and diminished T cell proliferation [36]. Interestingly, PD-L1, the predominant ligand for PD-1, is frequently found to be highly expressed in many types of cancer, and was shown to be upregulated under hypoxia [37-39]. To determine whether hypoxia also affects the expression of PD-1 on CD4⁺ effector T cells, and therefore could be involved in the reduced differentiation of CD4⁺ T cells into IFN-γ producing cells, we analysed the expression of PD-1 on CD4⁺Foxp3⁻ and CD4⁺Foxp3⁺ T cell in the colon of healthy control mice and mice suffering from CAC. Furthermore, we performed transcriptome analysis regarding the expression of PD-1 of CD4⁺Foxp3⁻ and CD4⁺Foxp3⁺ T cell cultured under hypoxic and normoxic conditions. Of note, we detected only a slight increase of PD-1 expression by CD4⁺Foxp3⁻ T cells in the colon of mice suffering from CAC compared to healthy control mice as well as after stimulation under hypoxia *in vitro* (Fig. 7A, B). In contrast, the expression of PD-1 was strongly enhanced on CD4⁺Foxp3⁺ Tregs during CAC and when exposed to hypoxia *in vitro* (Fig. 7A, B), which has been described to enhance the suppressive capacity of Tregs through the interaction

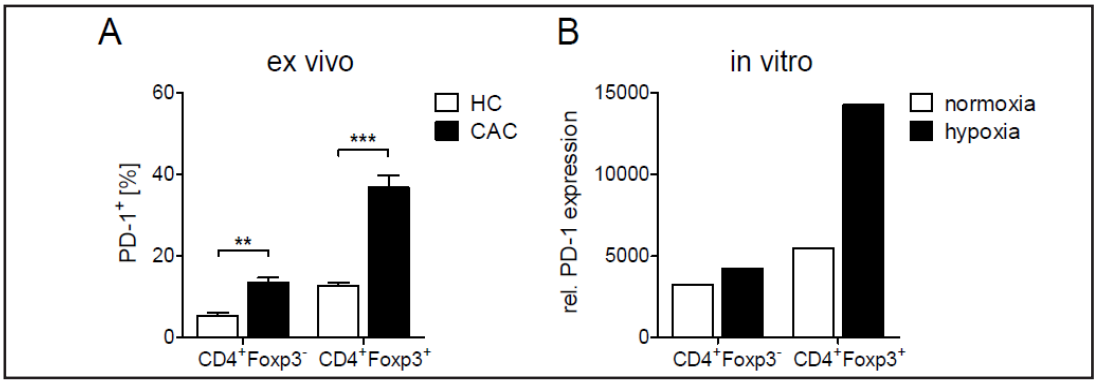
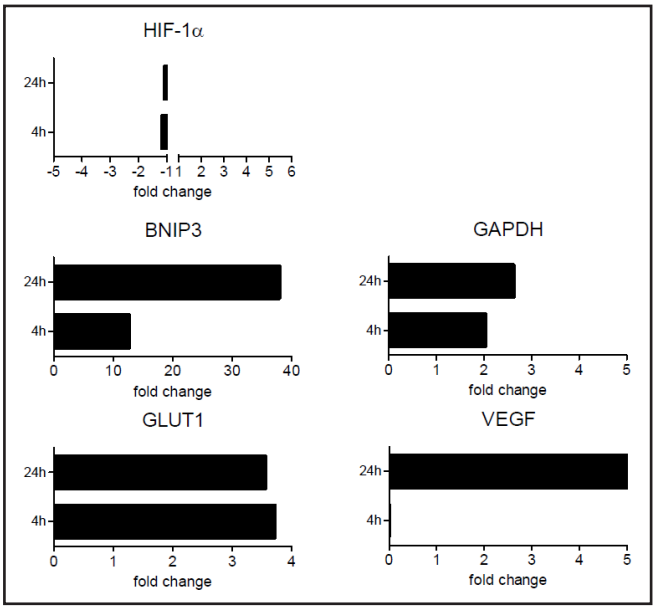


Fig. 7. Hypoxia strongly modulates the PD-1 expression of regulatory T cells. Colitis-associated colon cancer (CAC) was induced in BALB/c mice. (A) At week 12, LPLs from the colon were isolated and stained with antibodies against CD4, Foxp3, PD-1 and viability dye, and analysed by flow cytometry. Bar diagrams represent the percentage of PD-1 positive cells as mean ± SEM of n= 8 mice per group. Statistical analysis was performed with paired Student's t-test (** $p < 0.01$; *** $p < 0.001$). (B) Transcriptome data on PD-1 expression of $CD4^+Foxp3^-$ effector T cells and $CD4^+Foxp3^+$ Tregs stimulated *in vitro* under hypoxic and normoxic conditions.

Fig. 8. Expression of HIF-1α and selected hypoxia-inducible genes in regulatory T cells. Total $CD4^+Foxp3^+$ T cells were stimulated with αCD3/αCD28 under either hypoxic or normoxic conditions for 4 h or 24 h. RNA was isolated at indicated time points, transcribed into cDNA and used for quantitative real-time PCR. The expression was normalized to the housekeeping gene RPS9. The fold changes of the mean quantity of hypoxic versus normoxic samples are displayed.



with PD-L1 of effector T cells [40]. Thus, we identified that hypoxia enhances Treg-mediated immunosuppression rather than the induction of effector T cell exhaustion.

Again, we determined the impact of HIF-1α on the modulation of Treg function. Well in line with our former results, no difference in the expression of HIF-1α was detectable but the expression of BNIP3, GLUT1, GAPDH, and VEGF was strongly enhanced under hypoxic compared to normoxic conditions (Table 2, Fig. 8). Given the potential role of HIF-1α in regulating Treg function, it is not surprising that HIF-1α-deficient Tregs fail to control T-cell-mediated colitis [41].

Taken together, our data indicate that oxygen availability can function as a local modulator of immune responses in colitis-associated colon cancer. We observed severe colonic hypoxia in the cancerous crypts that was accompanied by a reduced $CD4^+$ effector T cell function and an enhanced suppressive activity of Tregs. Our complementary *in vitro* experiments show that the impaired T cell response can be referred to hypoxia. Nevertheless, we are aware that also other factors than hypoxia are involved in the inhibition of an effective anti-tumour

response *in vivo*. Further studies are needed to unravel whether pharmacologic strategies to induce HIF-1α stabilization offer the potential to shape the outcome of inflammation-associated colon cancer.

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Disclosure Statement

The authors declare no commercial or financial conflict of interest.

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