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ORIGINAL ARTICLE

In Mixed Lymphocyte Reaction, the Hypoxia-Inducible Factor Prolyl-Hydroxylase Inhibitor Roxadustat Suppresses Cellular and Humoral Alloimmunity

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Abstract

Hypoxia-inducible factor (HIF) prolyl-hydroxylase inhibitors are currently used for the treatment of renal anemia. Since HIF affects immune cells, we evaluated the effect of such a drug, the roxadustat, on adaptive immunity. Cell proliferation was assessed in a two-way mixed lymphocyte reaction (MLR) with BrdU assay. In $CD4^+$ T cells isolated from the two-way MLRs, western blotting was performed to detect the impact of roxadustat on HIF-1α and HIF-2α, the apoptotic marker cleaved caspase-3, and the master transcription factors of $CD4^+$ T cells differentiation towards Th1, Th2, Th17, Treg and Tfh subsets. The signature cytokines of the above $CD4^+$ T-cell subsets IFN-γ, IL-4, IL-17, IL-10, and IL-21 were measured in the supernatants. For assessing humoral immunity, we developed a suitable antibody-mediated complement-dependent cytotoxicity assay. Roxadustat stabilized HIF-1α and HIF-2α, suppressed cell proliferation, inhibited $CD4^+$ T-cell differentiation into Th1 and Th17 subsets, while it favored differentiation towards Th2, Treg and Tfh. Roxadustat suppressed humoral immunity too. These immunosuppressive properties of roxadustat indicate that the recently introduced HIF prolyl-hydroxylase inhibitors in medical therapeutics may render the patients vulnerable to infections. This possibility should be further evaluated in clinical trials.

Keywords Roxadustat · Hypoxia-inducible factor · Cellular immunity · Humoral immunity · T-cell differentiation

Introduction

Hypoxia-inducible factor (HIF) prolyl-hydroxylases (PH) inhibitors are a new class of drugs currently tested for the treatment of anemia in patients with chronic kidney disease (Gupta and Wish 2017). Roxadustat is a HIF-PH inhibitor, which has already completed the related phase III clinical trials and been approved for clinical use in China and Japan (Akizawa et al. 2020; Chen et al. 2019). By inhibiting PH, this class of drugs prevents HIF-2α hydroxylation and its subsequent E3 ubiquitin ligase Von Hippel–Lindau (VHL)-mediated ubiquitination and proteasomal degradation. The accumulated HIF-2α increases erythropoietin production (Gupta and Wish 2017). Also, HIF-PH inhibitors enhance HIF-1α level altering the expression of specific iron handling proteins, improving iron metabolism, and eventually, favoring erythropoiesis (Eleftheriadis et al. 2016a; Gupta and Wish 2017).

Besides genes implicated in erythropoiesis, HIF-1α and HIF-2α directly or indirectly affect the expression of many other genes involved, among others, in angiogenesis, cell metabolism, vasodilation, oxygen sensing, acid–base homeostasis and autophagy (Brahimi-Horn and Pouyssegur 2009; Choudhry and Harris 2018). Thus, the administration of PH inhibitors for the treatment of renal anemia may also alter the expression of other irrelevant to erythropoiesis genes raising concerns about possible side-effects. The possible overexpression of vascular endothelial growth factor A (VEGF-A), which is shown to enhance tumorigenesis or metastasis, is such a concern, and the performed clinical studies were too short term to exclude this possibility. However, PH inhibitors have not been found to increase circulating VEGF-A level in clinical studies (Brigandi et al. 2016; Holdstock et al. 2016), and animal studies support the safety

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of PH inhibitors as regards to oncogenesis and metastasis (Beck et al. 2017; Seeley et al. 2017).

Another aspect that has not been adequately studied is the effect of PH inhibitors on the adaptive immune response. Upon T-cell activation, the mammalian target of rapamycin complex 1 (mTORC1) increases HIF-1α level, even in the absence of hypoxia. HIF-1α alters T-cell function by affecting metabolism, proliferation, apoptosis, and differentiation towards various subtypes (Tao et al. 2015). Additionally, HIF-1α affects B-cell function directly and indirectly through its effect on helper T (Th) cells (Burrows and Maxwell 2017).

This study aimed to evaluate the effect of the PH inhibitor roxadustat on human adaptive immunity. Lymphocyte proliferation and differentiation of $CD4^+$ T cells into various subsets were assessed in the well-established model of two-way mixed lymphocyte reaction (MLR) (Lindemann 2014; Sato et al. 1999). For evaluating humoral immunity, we developed a suitable antibody-mediated complement-dependent cytotoxicity (CDC) assay-based protocol (Pissas and Eleftheriadis 2019).

Materials and Methods

Subjects

Ten healthy volunteers (33.6 ± 5.3 years old) enrolled in the study. Every volunteer had a free medical history, without any symptom, and physical examination and routine laboratory tests were normal. To exclude any pre-sensitization event, all subjects were males and without a history of blood transfusion, considering that pregnancy, blood transfusion, or previous transplantation may induce allo-sensitization (Hricik 2015). A written informed consent was obtained from each enrolled individual enrolled, and an official approval was received from the Ethics Committee of the Faculty of Medicine, University of Thessaly, Larissa, Greece (58/10–2-2017).

PBMCs Isolation and Culture

Blood was obtained via venipuncture, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation (Histopaque-1077; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). Isolated PBMCs were counted on a Neubauer chamber by inverse optical microscopy, and cell viability was assessed by trypan blue exclusion assay (Sigma-Aldrich; Merck Millipore). RPMI-1640 medium, with L-glutamine, and 10 mM HEPES (Sigma-Aldrich; Merck Millipore), and supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck Millipore) and antibiotic–antimycotic solution (Sigma-Aldrich; Merck Millipore) was used as culture medium. Cell cultures were performed at 37 °C in a humidified atmosphere (95%) and 5% CO_2.

Cytotoxicity Assay

For assessing the cytotoxicity of roxadustat, 1×10^5 resting PBMCs were cultured in 96-well plates for 7 days in the presence or not of roxadustat (Selleck Chemicals, Munich, Germany) at a concentration of 1, 5, 10 or 15 μg/mL. Roxadustat was dissolved in dimethyl sulfoxide (DMSO). An equal volume of DMSO was added in each well. Cytotoxicity was assessed by lactate dehydrogenase (LDH) release assay using the Cytotox Non-Radioactive Cytotoxic Assay kit (Promega Corporation, Madison, WI) according to the manufacturer's protocol and calculated by the equation Cytotoxicity (%) = (LDH in the supernatant: Total LDH) × 100. Ten independent experiments were performed, each one in triplicates. For the rest experiments of the study, the non-toxic roxadustat concentration of 5 μg/mL was used. Although this concentration corresponds to half of the serum C_{max} achieved after a single oral dose of 100 mg roxadustat (Groenendaal-van de Meent et al. 2016; Shibata et al. 2018), the unbound fraction of the drug used in the experiments was considerably higher than the respective fraction in the serum since the albumin concentration in the culture medium was significantly lower than in the serum.

Two-Way MLRs and Cell Proliferation

Adaptive immunity was evaluated by assessing cell proliferation in the well-established model of two-way MLR (Lindemann 2014; Sato et al. 1999). Two-way MLRs were performed in 96-well plates for 7 days in the presence or not of 5 μg/mL roxadustat. Roxadustat was dissolved in DMSO. An equal volume of DMSO was added as a vesicle control, whenever roxadustat was absent. The number of PBMCs from each member of the MLR couple was 5×10^4, summing up to a total of 1×10^5 PBMCs per well. Resting PBMCs cultured at a number of 1×10^5 per well were used as controls. At the end of the 7-day period, cell proliferation was assessed by Cell Proliferation ELISA (Roche Diagnostics, Indianapolis, IN, USA) using bromodeoxyuridine (BrdU) labeling and immunoenzymatic detection according to the protocol provided by the manufacturer. In this assay, BrdU is incorporated in the replicated DNA of proliferating cells, and the amount of the incorporated BrdU assessed immunoenzymatically using anti-BrdU antibodies corresponds to the degree of proliferation (Huong et al. 1991; Muul et al. 2011). The proliferation index was calculated according to the equation Proliferation index = optical density derived from each MLR: the optical density of the control. According to the BrdU assay protocol provided by the manufacturer,

the control in a two-way MLR corresponds to the mean of the optical densities derived from the resting PBMCs cultures of the two subjects that constituted the specific MLR (Muul et al. 2011). Ten independent MLRs were performed, each one in triplicates.

Isolation of CD4$^+$ T Cells from Two-Way MLRs and Assessment of the Proteins of Interest

The impact of roxadustat on CD4$^+$ T-cell apoptosis, as well as on CD4$^+$ T-cell differentiation towards various effector subtypes or regulatory T cells (Tregs) was evaluated in CD4$^+$ T-cell isolated from two-way MLRs.

Two-way MLRs were performed in 24-well plates for 7 days in the presence or not of 5 μg/mL roxadustat. Roxadustat was dissolved in DMSO. An equal volume of DMSO was added as a vesicle control, whenever roxadustat was absent. The number of PBMCs from each member of the MLR couple was 5×10^5, summing up to a total of 1×10^6 PBMCs per well. At the end of the 7-day period, CD4$^+$ T cells were isolated from the two-way MLRs by negative selection using the CD4$^+$ T-Cell Isolation Kit, Human (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Flow cytometry revealed that the mean purity of isolated CD4$^+$ T cells was equal to 97%. Isolated CD4$^+$ T cells were counted on a Neubauer chamber by inverse optical microscopy, and cell viability was determined by trypan blue exclusion assay.

Equal numbers of CD4$^+$ T cells were lysed using the T-PER tissue protein extraction reagent (Thermo Fisher Scientific Inc., Rockford, IL, USA) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich; Merck Millipore and Roche Diagnostics, respectively). Bradford assay (Sigma-Aldrich; Merck Millipore) was used for protein quantification, and 10 μg from each sample were loaded for western blotting. Blots were incubated with the primary antibody for 16 h, and then for 30 min with the secondary antibody. In the case of reprobing polyvinylidene fluoride blots, the Restore Western Blot Stripping Buffer (Thermo Fisher Scientific Inc.) was applied. The Image J software (National Institute of Health, Bethesda, MD, USA) was used for analyzing the western blots.

The primary antibodies used in western blotting were specific against HIF-1α (dilution 1:500, cat. no. 3716; Cell Signaling Technology, Danvers, MA, USA), HIF-2α (dilution 1:500, cat. no. 7096; Cell Signaling Technology), cleaved caspase-3 (dilution 1:1000, cat. no. 9664; Cell Signaling Technology), T-box transcription factor TBX21 (T-bet; dilution 1:500, cat. no. 13232; Cell Signaling Technology), trans-acting T-cell-specific transcription factor GATA-3 (GATA-3; dilution 1:500, cat. no. 5852; Cell Signaling Technology), retinoic acid receptor-related orphan receptor-γt (RORγt; dilution 1:500, cat. no. orb6888; Biorbyt, Cambridge, UK), Forkhead box P3 (FoxP3; dilution 1:500, cat. no. 5298; Cell Signaling Technology), B-cell lymphoma 6 protein (Bcl-6; dilution 1:500, cat. no. E-AB-15965; Elabscience, Houston, TX), and β-actin (dilution 1:5000, cat. no. 4967; Cell Signaling Technology). An anti-rabbit IgG, HRP-linked antibody (cat. no. 7074; Cell Signaling Technology) was used as the secondary antibody.

Cytokines in the Supernatants of Two-Way MLRs

Two-way MLRs were performed in 24-well plates for 7 days in the presence or not of 5 μg/mL roxadustat. Roxadustat was dissolved in DMSO. An equal volume of DMSO was added as a vesicle control, whenever roxadustat was absent. The number of PBMCs from each member of the MLR couple was 5×10^5, summing up to a total of 1×10^6 PBMCs per well. At the end of the 7-day period, supernatants were collected and stored at − 80 °C. Ten independent MLRs were performed. The signature cytokines of the Th1, Th2, Th17 cells, Treg, and follicular helper T (Tfh) cells were measured in the supernatants with ELISA.

For this purpose, the Human IFN-γ Quantikine ELISA Kit (sensitivity less than 8 pg/mL, R&D Systems, Minneapolis, MN, USA), the Human IL-4 Quantikine ELISA Kit (sensitivity less than 10 pg/mL, R&D Systems), the Human IL-17 Quantikine ELISA Kit (sensitivity less than 15 pg/mL, R&D Systems), the Human IL-10 Quantikine ELISA Kit (sensitivity less than 3.9 pg/mL, R&D Systems), and the Human IL-21 ELISA kit (sensitivity 0.78 pg/mL, Biorbyt, Cambridge, UK) were used.

One-Way MLRs and Assessment of Humoral Immunity

We developed a protocol for assessing the effect of roxadustat on humoral immunity. The time span of 1 week that an MLR lasts is proven sufficient for antibody production (Rumke et al. 1982). Supernatants from one-way MLRs contain antibodies produced by the responder PBMCs against the stimulator PBMCs. These supernatants were used in antibody-mediated CDC assay against resting target PBMCs of the same origin as those used as stimulator cells in the respective one-way MLRs. We validated this protocol in previous studies (Eleftheriadis et al. 2017a, b, c).

In one-way MLRs, mitomycin C-treated PBMCs (Sigma Aldrich; Merck Millipore) were used as stimulator cells. For the mitomycin C treatment, PBMCs were incubated with 50 μg/mL mitomycin C for 30 min at 37 °C and then washed three times with complete RPMI-1640. One-way MLRs were performed in 24-well plates. To the 5×10^5 stimulator cells, 5×10^5 PBMCs from another individual

were added as responder cells. One-way MLRs lasted for 7 days in the presence or not of 5 µg/mL roxadustat. Roxadustat was dissolved in DMSO. An equal volume of DMSO was added as a vesicle control, whenever roxadustat was absent. At the end of the 7-day period, supernatants containing the antibodies produced by the responder PMBCs against the stimulator mitomycin C-treated PBMCs were collected. Ten independent one-way MLRs were performed.

In parallel with the one-way MLRs, resting untreated PBMCs were cultured in six-well plates. Once the one-way MLRs were over, the resting PBMCs of the same origin as those used as stimulator cells in one-way MLRs were counted and plated in 96-well plates at a number of 5×10^4 and in a volume of 50 µL to serve as target cells in an antibody-mediated CDC assay. A volume of 50 µL from each supernatant per respective one-way MLR was added into the 96-well plates already seeded with the resting target PBMCs. The plates were incubated for 30 min on ice. Then, 11 µL of rabbit complement (Low-Tox-H rabbit complement, Cedarlane Corporation, Burlington, Ontario, Canada) were added to each well, and the 96-well plates were incubated for another 2 h at 37 °C. As a control, 50 µL of complete RPMI-1640 instead of the one-way MLR supernatant were added to resting target PBMCs, and after incubation for 30 min on ice, 11 µL of rabbit complement was added for another 2 h incubation at 37 °C.

The TACS XTT assay kit (Trevigen, Gaithersburg, MD, USA) was used for assessing the survival of the target PBMCs according to the manufacturer's protocol. Target cells were incubated with the XTT reagent for 1 h, and cell survival was calculated by the equation Cell survival (%) = (XTT assay OD of the control / XTT assay OD of the evaluated condition) × 100. Since ten independent one-way MLRs were performed, ten antibody-mediated CDC experiments were performed as well, each one of the latter in triplicates.

Statistical Analysis

Statistical analysis was performed with the IBM SPSS Statistics for Windows, version 20 (IBM Corp., Armonk, NY, USA). One sample Kolmogorov–Smirnov test confirmed the normality of the evaluated variables. For comparison of means, paired *t* test was used. Whenever more than two groups were compared, the repeated-measures ANOVA followed by Bonferroni's correction test was used. Results were expressed and depicted as mean ± standard error of mean (SEM), and $p < 0.05$ was considered as statistically significant. For the reader's convenience, most of the results were presented after normalization of means for the control group of untreated cells.

Results

Cytotoxicity of Roxadustat in Resting PBMCs

Cytotoxicity of roxadustat on resting PMBCs was tested first. LDH-release assay revealed that treatment of resting PBMCs for 1 week with roxadustat at concentrations of 1, 5 or 10 µg/mL was not toxic. However, at the concentration of 15 µg/mL, roxadustat was toxic (Fig. 1). We selected the non-toxic concentration of 5 µg/mL roxadustat for the rest experiments. This concentration corresponds to approximately half of the C_{max} observed after the administration of a single 100 mg oral dose of roxadustat (Groenendaal-van de Meent et al. 2016; Shibata et al. 2018). However, because cell culture medium albumin is lower than in the serum, the used unbound fraction of roxadustat was considerably higher than that observed in pharmacokinetic studies.

The Effect of Roxadustat on the Level of HIF-1α and HIF-2α in CD4$^+$ T Cells Isolated from Two-Way MLRs

To verify the expected pharmacodynamic effect of roxadustat, CD4$^+$ T-cell were isolated from two-way MLRs. Indeed, western blotting confirmed that roxadustat increases the levels of HIF-1α and HIF-2α in CD4$^+$ T cells significantly, by a factor of 2.5 and 1.8, respectively (Fig. 2).

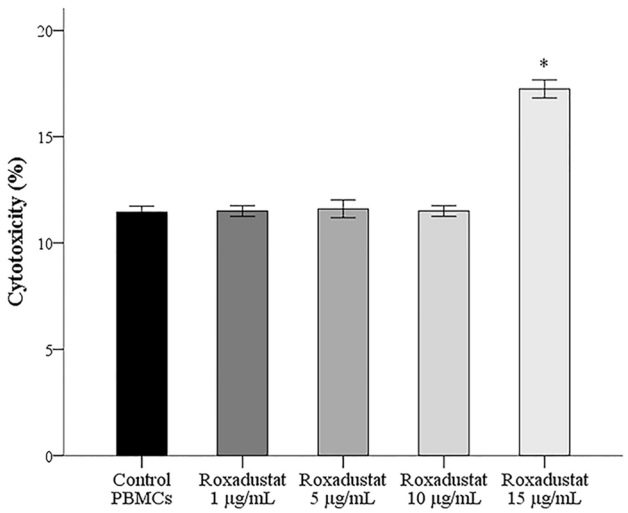

Fig. 1 Cytotoxicity of roxadustat in resting PBMCs. LDH release assay showed that roxadustat at concentrations of 1, 5, or 10 µg/mL is not cytotoxic for resting PBMCs. On the contrary, 15 µg/mL roxadustat proved to be cytotoxic. Ten independent experiments were performed, each one in triplicates. Error bars correspond to the SEM, and asterisk to a $p < 0.001$ compared to all the other conditions

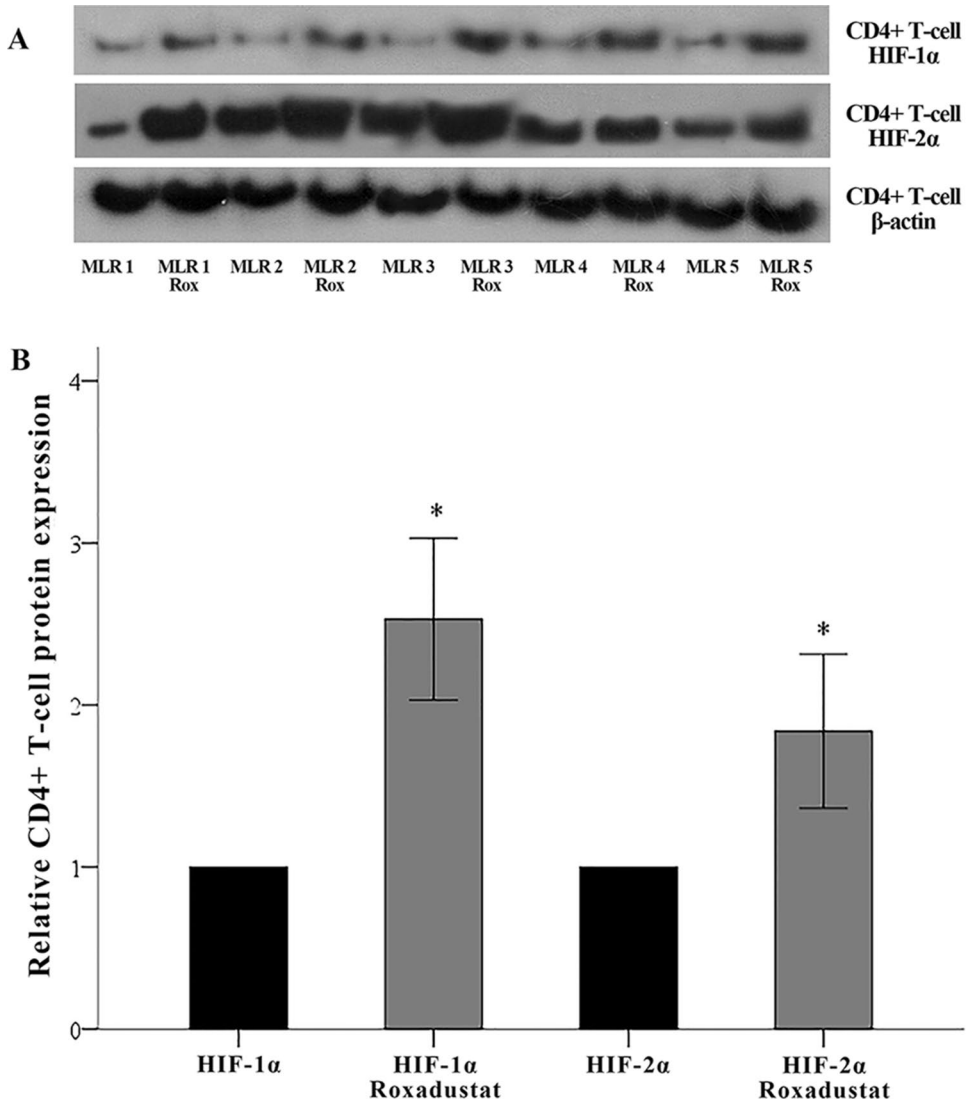

Fig. 2 The effect of roxadustat on the level of HIF1α and HIF2α in $CD4^+$ T cells isolated from two-way MLRs. Ten independent two-way MLRs were performed in the presence or not of roxadustat (5 µg/mL). After 1 week, $CD4^+$ T cells were isolated, and western blotting was performed for assessing HIF-1α and HIF-2α levels. **a** Depicts the results from five out of the ten performed experiments. As shown in (**b**), roxadustat increased both HIF-1α and HIF-2α. The error bars correspond to the SEM, and the asterisks to a $p < 0.001$ compared to the untreated cells

The Effect of Roxadustat on Cell Proliferation in Two-Way MLRs, and Apoptosis in $CD4^+$ T Cells Isolated from the Two-Way MLRs

Experiments in two-way MLRs showed that roxadustat significantly suppresses cell proliferation. Since clonal expansion follows antigen-specific lymphocyte stimulation (Abbas and Janeway 2000), this result indicates that roxadustat downregulates adaptive immunity. Compared to the control resting PBMCs, in MLRs, the proliferation index was about two times (2.04) higher. In contrast, the proliferation index in the roxadustat-treated MLRs was almost the same (1.03) as in control (Fig. 3a).

Cell apoptosis in $CD4^+$ T cells isolated from two-way MLRs was evaluated by the level of activated cleaved caspase-3 in which all the apoptotic pathways converge (Fadeel and Orrenius 2005). Roxadustat increased apoptosis in $CD4^+$ T cells. Compared to the $CD4^+$ T cells derived from untreated MLRs, the level of cleaved caspase-3 in $CD4^+$ T cells isolated from roxadustat-treated MLRs was 1.6 times higher (Fig. 3b, c).

The Effect of Roxadustat on the Master Transcription Factors that Regulate $CD4^+$ T-Cell Differentiation towards Various Subsets

The effect of roxadustat on MLR-derived $CD4^+$ T-cell differentiation into various subsets was evaluated by the expression of the master transcription factors that control this process (Fang and Zhu 2017; Saravia et al. 2019).

Compared to untreated cells, roxadustat decreased T-bet level, the master transcription factor of Th1, almost by half (0.47). On the contrary, $CD4^+$ T cells isolated from roxadustat-treated two-way MLRs exhibited an almost twofold (1.83) increase in GATA-3 expression, the master transcription factor of Th2 (Fig. 4a, b).

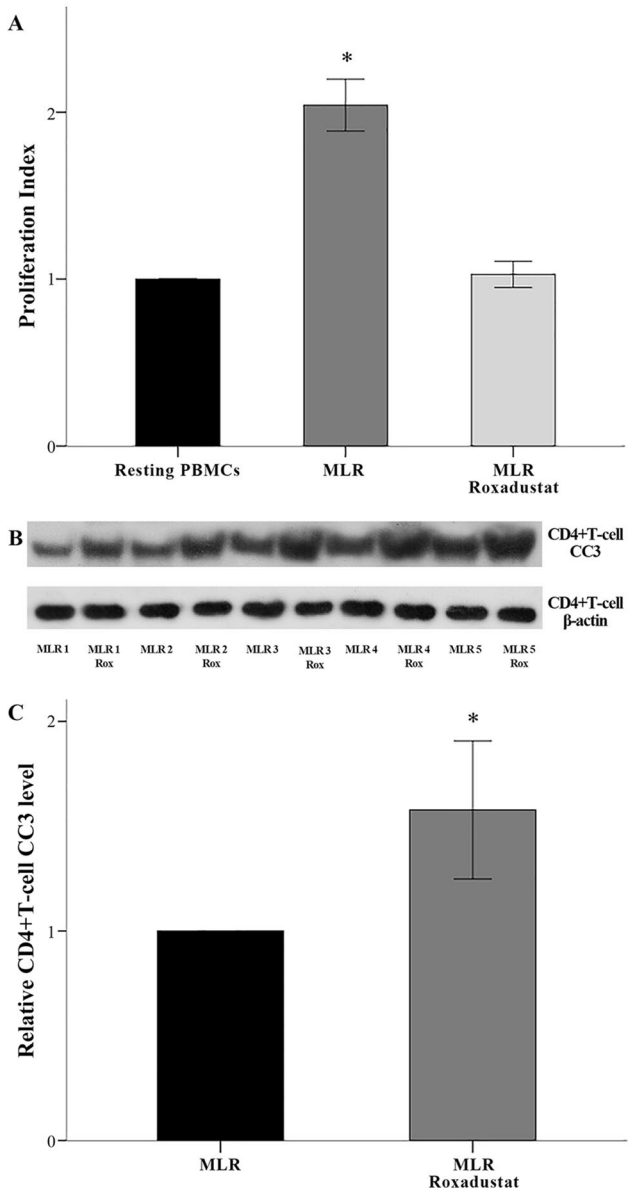

Fig. 3 The effect of roxadustat on cell proliferation in two-way MLRs, and apoptosis in $CD4^+$ T cells isolated from the two-way MLRs. Ten independent two-way MLRs were performed in 96-well plates in the presence or not of roxadustat (5 μg/mL), each one in triplicates. BrdU assay revealed that roxadustat decreased cell proliferation significantly (**a**). Ten independent two-way MLRs were performed in 24-well plates in the presence or not of roxadustat (5 μg/mL). After 1 week, $CD4^+$ T cells were isolated, and western blotting was performed for assessing the marker of cell apoptosis cleaved caspase-3. **b** Depicts the results from five out of the ten performed experiments. As shown in panel C, roxadustat increased the level of cleaved caspase-3. CC3 stands for cleaved caspase-3. The error bars correspond to the SEM, and the asterisk to a $p < 0.001$ compared to the untreated cells

In $CD4^+$ T cells derived from roxadustat-treated MLRs, the expression of RORγt, the master transcription factor of Th17, was reduced almost to the half (0.54) of the level detected in untreated cells. The effect of roxadustat on the expression of FoxP3, the master transcription factor of Treg was the opposite. Roxadustat nearly doubles (1.86) the FoxP3 levels (Fig. 4a, b).

Finally, the expression levels of the master regulator transcription factor of Tfh, Bcl-6, were also examined. Indeed, in the context of $CD4^+$ T cells derived from roxadustat-treated MLRs, the expression of the Bcl-6 transcription factor was more than twofold (2.27) higher than that detected in $CD4^+$ T cells derived from untreated MLRs (Fig. 4a, b).

The Effect of Roxadustat on the Signature Cytokines of the $CD4^+$ T-Cell Subsets

In addition to the master transcription factors that regulate $CD4^+$ T-cell differentiation into various subsets, the interferon (IFN)-γ, interleukin (IL)-4, Il-17, IL-10, and IL-21, which are signature cytokines of the Th1, Th2, Th17, Treg, and Tfh subsets, respectively (Loo et al. 2018), were measured in the supernatants of the two-way MLRs.

Roxadustat reduced IFN-γ (58 ± 4.2 pg/mL vs. 126.5 ± 5.7 pg/mL, $p < 0.001$) and IL-17 (114.3 ± 4.9 pg/mL vs. 230.4 ± 9.8 pg/mL, $p < 0.001$) concentrations. On the contrary, roxadustat enhanced the level of IL-4 (187.1 ± 4.1 pg/mL vs. 115.1 ± 7.3 pg/mL, $p < 0.001$) and IL-10 (133 ± 7.5 pg/mL vs. 63.8 ± 2 pg/mL, $p < 0.001$). The concentration of IL-21 remained unaffected by roxadustat treatment (99.6 ± 5.7 pg/mL vs. 93.3 ± 4.3 pg/mL, p not significant) (Fig. 5).

The Effect of Roxadustat on the Humoral Immune Response

To assess humoral immunity, one-way MLR was set up. In one-way MLR, responder B cells, which is a fraction of the responder PBMCs, are differentiated into plasma cells and produce antibodies against the stimulator PBMCs (Rumke et al. 1982). Supernatants of these MLRs contained such antibodies and were used in an antibody-mediated CDC assay against resting target PMBCs of the same origin as the stimulator PBMCs of the respective MLRs.

In MLRs, the cellular immune response inevitably results in some degree of cell death (Sato et al. 1999), and consequently, in the release of LDH. Since roxadustat alters the intensity of the MLRs and, therefore, the amount of the released LDH, we did not perform the cytotoxicity LDH release assay, but instead, we assessed cellular survival through the XTT assay. Supernatants from untreated MLRs decreased the survival of target PBMCs to 66% of the control ($p < 0.001$), whereas supernatants from roxadustat-treated MLRs did not affect cell survival (113% of the control, p not significant) (Fig. 6). Thus, roxadustat inhibited antibody production.

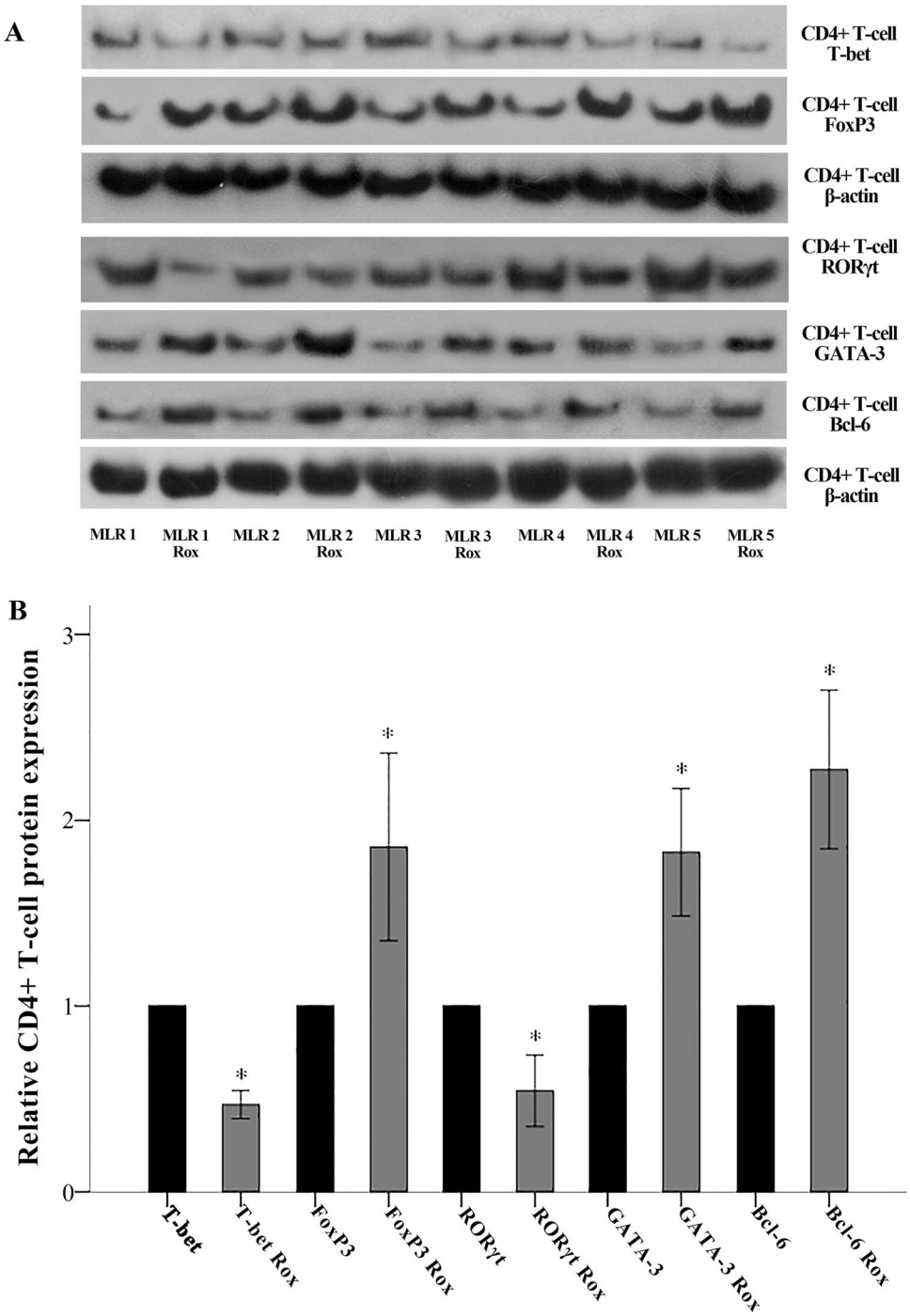

Fig. 4 The effect of roxadustat on the master transcription factors that regulate CD4 + T-cell differentiation towards various subsets. Ten independent two-way MLRs were performed in the presence or not of roxadustat (5 μg/mL). After 1 week, $CD4^+$ T cells were isolated and western blotting was performed for evaluating the expression of the master transcription factors T-bet, GATA-3, RORγt, FoxP3, and Bcl-6, that regulate $CD4^+$ T-cell differentiation towards Th1, Th2, Th17, Treg, and Tfh subset, respectively. **a** Depicts the results from five out of the ten performed experiments. As shown in (**b**), roxadustat decreased the expression of T-bet and RORγt, whereas it increased the expression of GATA-3, FoxP3, and Bcl-6. The error bars correspond to the SEM, and the asterisks to a $p < 0.001$ compared to the untreated cells

Discussion

Considering that HIF-1α affects lymphocyte metabolism, survival, proliferation, as well as $CD4^+$ T-cell differentiation towards different subsets (Burrows and Maxwell 2017; Tao et al. 2015), we evaluated the effect of roxadustat, a PH inhibitor currently used for the treatment of renal anemia (Akizawa et al. 2020; Chen et al. 2019), on the adaptive immunity.

Primary human PBMCs and roxadustat at the concentration of 5 μg/mL were used. This concentration corresponds to approximately half of the C_{max} achieved after administration of a single oral dose of 100 mg roxadustat (Groenendaal-van de Meent et al. 2016; Shibata et al. 2018). However, as already noted, the unbound fraction of roxadustat used in the experiments was significantly higher than the unbound fraction observed in pharmacokinetic studies. Notably, at the concentration of 5 μg/mL, roxadustat was

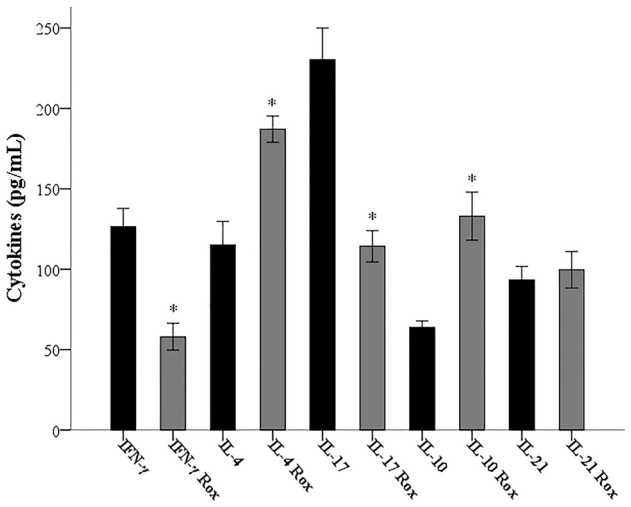

Fig. 5 The effect of roxadustat on the signature cytokines of the $CD4^+$ T-cell subsets. Ten independent two-way MLRs were performed in the presence or not of roxadustat (5 μg/mL). After 1 week, supernatants were collected, and the concentration signature cytokines for Th1, Th2, Th17, Treg, and Tfh, which are IFN-γ, IL4, IL-17, IL-10, and IL-21 respectively were measured with ELISA. Roxadustat decreased IFN-γ and IL-17 concentration, increased the level of IL-4 and IL-10, but left IL-21 unaffected. The error bars correspond to the SEM, and the asterisks to a $p < 0.001$ compared to the untreated cells

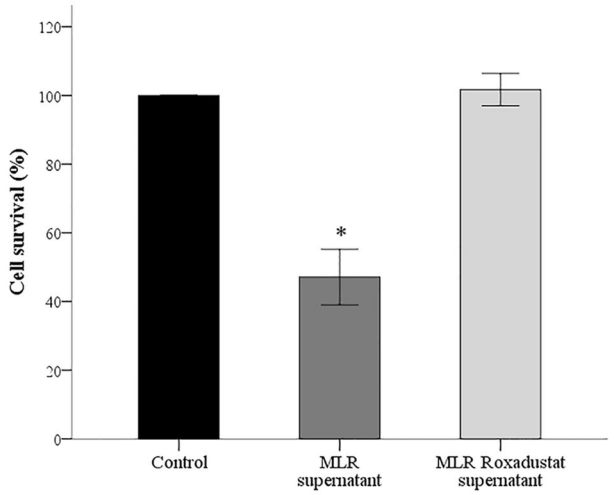

Fig. 6 The effect of roxadustat on the humoral immune response. Ten independent one-way MLRs were performed in the presence or not of roxadustat (5 μg/mL). After 1week, supernatants were collected and used in an antibody-mediated CDC assay against resting target PBMCs of the same origin as the respective stimulator cells of the MLRs. XTT assay was used for assessing cell survival and revealed that contrary to supernatants from untreated MLRs, supernatants from roxadustat treated MLRs did not decrease cell survival, indicating that roxadustat suppresses humoral immunity. The error bars correspond to the SEM, and the asterisk to a $p < 0.001$ compared to all the other conditions

not toxic for resting PBMCs and increased both HIF-1α and HIF-2α levels in $CD4^+$ T cells isolated from two-way MLRs.

In two-way MLRs, roxadustat decreased cell proliferation. Since clonal expansion follows antigen-specific lymphocyte stimulation (Abbas and Janeway 2000), this result indicates that roxadustat downregulates adaptive immunity. The observed increased $CD4^+$ T-cell apoptosis may contribute to the above effect. It is known that HIF-1α can induce cell-death through various molecular pathways (Harris 2002). Another possible explanation could be the differentiation of $CD4^+$ T cells into Treg since, in our study, HIF-1α stabilization increased the expression of FoxP3, the master transcription factor of Treg. Treg inhibit antigen-induced effector T-cell proliferation (Fang and Zhu 2017; Saravia et al. 2019). Finally, the effect of HIF-1α stabilization on cell metabolism cannot be ignored. HIF shifts cell metabolism towards glycolysis instead of Krebs cycle and oxidative phosphorylation. Although the role of aerobic glycolysis in the proliferation of activated $CD4^+$ T cells is emphasized by many investigators, oxidative phosphorylation is also necessary (Caro-Maldonado et al. 2012; Chang et al. 2013; Eleftheriadis et al. 2018; Roos and Loos 1973; Sena et al. 2013; Wang and Green 2012; Yin et al. 2016). Interestingly, in two-way MLRs with human PBMCs, the aerobic glycolysis inhibitor dichloroacetate does not affect cell proliferation, whereas the Krebs cycle inhibitor LW6 inhibits cell proliferation (Eleftheriadis et al. 2017b).

Regarding $CD4^+$ T-cell differentiation towards various subsets, roxadustat had a profound effect, as it altered the expression of all the evaluated master transcription factors that determine the $CD4^+$ T-cell subsets. The first discovered $CD4^+$ T cells subsets were the Th1 and Th2 (Saravia et al. 2019). Th1 by activating macrophages are responsible for the elimination of intracellular pathogens, whereas Th2 by activating eosinophils and mast cells and by inducing antibody class switching to IgE are responsible for immunity against extracellular parasites (Fang and Zhu 2017; Saravia et al. 2019). According to the initial Th1/Th2 paradigm (Romagnani 1997), $CD4^+$ T-cell differentiation into Th1 inhibits differentiation into Th2 and vice versa (Fang and Zhu 2017; Romagnani 1997; Saravia et al. 2019). In our model, roxadustat decreased T-bet, the master transcription factor of Th1 (Fang and Zhu 2017; Saravia et al. 2019), and in parallel, it increased GATA-3, the master transcription factor of Th2 (Fang and Zhu 2017; Saravia et al. 2019). In addition, roxadustat reduced IFN-γ but enhanced IL-4 level in the MLR supernatants, also supporting that roxadustat inhibits $CD4^+$ T-cell differentiation into Th1 cells but promotes differentiation into Th2 cells (Loo et al. 2018). Our results agree with studies showing that hypoxia and HIF stabilization in $CD4^+$ T cells enhances Th2 and reduces Th1 (Saini et al. 2010; Westendorf et al. 2017). Another possible explanation, not evaluated in the present study, may rely on

the effect of roxadustat on macrophages. Hypoxia and HIF stabilization favor macrophages differentiation towards M2 instead of M1 phenotype (Leblond et al. 2015; Rahat et al. 2011; Yang et al. 2010). M2 macrophages induce $CD4^+$ T-cell differentiation into Th2, while M1 macrophages into Th1 (Biswas and Mantovani 2010).

Following the initial discovery of Th1 and Th2, the Treg and Th17 subsets were identified (Saravia et al. 2019). Th17 recruit and activate neutrophils helping in the elimination of extracellular bacteria, while Treg suppress and regulate the adaptive immune response (Fang and Zhu 2017; Saravia et al. 2019). As with the Th1/Th2 paradigm (Romagnani 1997), the Th17/Treg paradigm supports that $CD4^+$ T-cell differentiation into Th17 inhibits differentiation into Treg and vice versa (Fang and Zhu 2017; Jadidi-Niaragh and Mirshafiey 2012; Saravia et al. 2019). In our model, roxadustat decreased RORγt, the master transcription factor of Th17 (Fang and Zhu 2017; Saravia et al. 2019), and in parallel, increased FoxP3, the master transcription factor of Treg (Fang and Zhu 2017; Saravia et al. 2019). The fact that IL-17 concentration decreased, while IL-10 level increased in the supernatants from the roxadustat-treated MLRs, is in agreement with the alterations in the expression of RORγt and FoxP3, respectively (Loo et al. 2018). There is a discrepancy in the literature regarding the effect of HIF on Th17 and Treg differentiation, with some studies supporting that HIF1α stabilization increases Th17 and decreases Treg (Dang et al. 2011; Shi et al. 2011), and with other studies claiming the opposite (Ben-Shoshan et al. 2008; Clambey et al. 2012). However, many of these studies performed in non-human murine T cells (Ben-Shoshan et al. 2008; Clambey et al. 2012; Dang et al. 2011; Shi et al. 2011). Importantly, in those studies, in vitro experiments were performed in cultured isolated $CD4^+$ T cells treated with certain T-cell subset skewing cytokines. Instead we used the MLR model. MLR partly mimics the in vivo situation since it allows the interaction of the various cells of the immune system and the de novo production of the cytokines required for $CD4^+$ T-cell differentiation into various subsets (Eleftheriadis et al. 2016b; Lindemann 2014; Rumke et al. 1982; Sato et al. 1999). Finally, in the cited studies, the various methods used for affecting HIF1α level mostly involved genetic depletion of HIF-1α or VHL. Interestingly, a group that also used a PH inhibitor for stabilizing HIF-1α, drew the same conclusion as the present study, that HIF stabilization favors $CD4^+$ T-cell differentiation into Treg instead of Th17 (Clambey et al. 2012). Collectively and according to the Th1/Th2 and the Th17/Treg paradigms, roxadustat exerts mostly immunosuppressive effects as regards $CD4^+$ T-cell differentiation.

Following the discovery of the Th1, Th2, Th17, and Treg subsets, another $CD4^+$ T-cell subset was detected, the Tfh (Saravia et al. 2019). After differentiation into the lymph nodes, and contrary to the already mentioned $CD4^+$ T-cell subsets, Tfh are not directed to the region of the inflammatory response, but they migrate into the lymph node germinal centers where they provide help to B cells to become antibody-secreted long-lived plasma cells or memory B cells (Fang and Zhu 2017; Saravia et al. 2019). There is a discrepancy in the effect of hypoxia and HIF stabilization on $CD4^+$ T-cell differentiation into Tfh. For instance, in one study, HIF stabilization through genetic VHL depletion enhanced differentiation into Tfh (Abbott et al. 2016), while in another the same manipulation led to the opposite (Zhu et al. 2019). In our model, in isolated from two-way MLR $CD4^+$ T cells, roxadustat increased the signature transcription factor of Tfh subset Bcl-6. However, MLR treatment with roxadustat did not increase IL-21 concentration in the supernatant. Although IL-21 is the signature cytokine of Tfh (Loo et al. 2018), Th17 cells also produce significant amounts of IL-21 (Loo et al. 2018; Wei et al. 2007), and as already noted roxadustat inhibits $CD4^+$ T-cell differentiation into Th17 cells.

Although in our experiments roxadustat induced Bcl-6 expression, humoral immunity was suppressed. At first glance, this is a paradox; however, there are some possible explanations. The reason may rely on the general suppressive effect of roxadustat on T cells, which provide help to B cells. The direct impact of roxadustat on HIF stabilization in B cells could also play a role. Stabilization of HIF-1α by B-cell-specific depletion of VHL inhibits mTORC1 activity in B lymphoblasts, and mTORC1-haploinsufficient B cells have reduced clonal expansion, activation-induced cytosine deaminase expression, and capacities to yield IgG2c and high-affinity antibodies (Cho et al. 2016). Another possible explanation may rely on cell metabolism. Although B-cell metabolism is less well studied than T-cell metabolism (Caro-Maldonado et al. 2012), activated B cells utilize both aerobic glycolysis and oxidative phosphorylation (Caro-Maldonado et al. 2014; Dufort et al. 2014; Garcia-Manteiga et al. 2011), and plasma cells are mostly dependent on oxidative phosphorylation for covering their high energy demands for antibody production (Garcia-Manteiga et al. 2011; Lam et al. 2016; Shaffer et al. 2004). Thus, by shifting cell metabolism towards aerobic glycolysis instead of Krebs cycle and oxidative phosphorylation, HIF may suppress plasma cell antibody production. Interestingly, in an experimental model similar to the present study, in which the aerobic glycolysis inhibitor DCA was compared to the Krebs cycle inhibitor LW6, only LW6 suppressed antibody production (Eleftheriadis et al. 2017b). Regardless of the responsible molecular mechanism, our results support that roxadustat suppresses humoral immunity.

Collectively, the results of our study are presented in Fig. 7. A limitation of our study lies in the fact that we did not evaluate in-depth the molecular mechanisms that mediate the immunosuppressive effects of roxadustat. However,

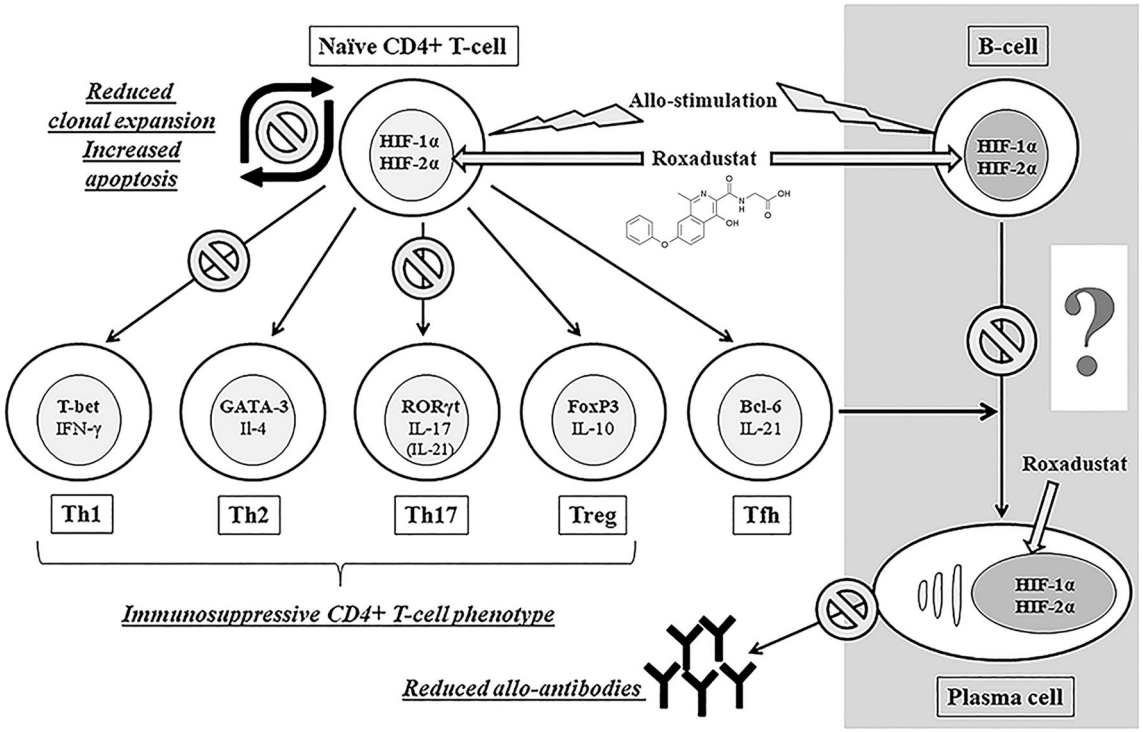

Fig. 7 The effect of roxadustat on adaptive immunity. In two-way MLRs, roxadustat inhibits lymphocyte clonal expansion and causes $CD4^+$ T-cell apoptosis. Also, it induces $CD4^+$ T-cell differentiation towards Th2, Treg, and Tfh subsets, whereas in inhibits differentiation into Th1, and Th17 subsets. In addition to promoting an immunosuppressive $CD4^+$ T-cell phenotype, roxadustat inhibits alloantibody production. The gray area of the figure corresponds to processes that were not investigated in the present study, but are strongly supported by the cited literature

our primary aim was to assess the impact of roxadustat on adaptive immunity by evaluating specific end-points of $CD4^+$ T-cell activation and differentiation, and antibody production since PH inhibitors are introduced in the treatment of anemia of chronic kidney disease very recently. Also, as already noted, in our experiments, the unbound to protein roxadustat concentration was significantly higher than that observed in pharmacokinetic studies. Finally, from all PH inhibitors, only roxadustat was evaluated. Consequently, our results may not be applied with certainty to other available PH inhibitors.

In conclusion, roxadustat, a PH inhibitor used for the treatment of renal anemia, suppresses T-cell clonal expansion, induces $CD4^+$ T-cell apoptosis, skewing $CD4^+$ T-cell differentiation towards an immunosuppressive phenotype. Furthermore, roxadustat suppresses humoral immunity. Such immunosuppressive properties of roxadustat, and possibly of other PH inhibitors as well, should be further evaluated in clinical trials. Although phase III clinical trials support the safety of roxadustat, the observed increased incidence of upper respiratory tract infection may reflect the roxadustat-induced immunosuppression (Akizawa et al. 2020; Chen et al. 2019). The situation might get worse in the case of concurrent diseases that suppress the immune system or co-administration of other immunosuppressive medications.

Author contributions TE designed the study; GP, and TE performed all the experiments except the determination of CD4 + T-cell purity by flow cytometry, which was performed by AM; TE, GP, EN, GF, and VL analyzed the results; TE wrote the manuscript with help from GP; IS supported all stages; all authors approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest.

Research Involving Human Participants and Animals Informed consent was obtained from each individual enrolled in the study, and official approval was received from the Ethics Committee of the Faculty of Medicine, University of Thessaly, Larissa, Greece (58/10-2-2017).

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