Dynamic regulation of Th17 differentiation by oxygen concentrations

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Received 15 September 2011, accepted 1 December 2011

Abstract

Naive CD4⁺ T cells are activated by antigen-presenting cells (APCs) and differentiate into distinct types of helper T (Th) cells in the lymph node or spleen. Oxygen (O₂) tension is generally low in these secondary lymphoid tissues compared with the bloodstream or atmosphere. However, the effect of changes in O₂ concentration on the differentiation of Th cells remains unclear. Here, we established a novel model of Th-cell differentiation, which mimics physiological O₂ conditions. We primed naïve CD4⁺ T cells under 5% O₂, which has been observed in the lymph node or spleen and reoxygenated under normoxia that mimicked the O₂ concentration in blood. In this model, the differentiation of Th17 cells, but not Th1 or iTreg cells, was enhanced. Under the condition of 5% O₂, mammalian target of rapamycin complex 1 (mTORC1) was activated and led to the stabilization of hypoxia-inducible factor 1α (HIF-1α) in Th17 cells. The activation of mTORC1 and the acceleration of Th17-cell differentiation, which occurred when cells were primed under 5% O₂, were not observed in the absence of HIF-1α but were accelerated in the absence of von Hippel–Lindau tumor suppressor protein (vHL), a factor critical for HIF-1α degradation. Thus, a positive feedback loop between HIF-1α and mTORC1 induced by hypoxia followed by reoxygenation accelerates Th17-cell differentiation.

Keywords: HIF-1α, IL-17, mTORC1, reoxygenation

Introduction

O₂ tension in tissues and organs is generally much lower than that found in the atmosphere. In addition, inflamed and cancerous tissues show even lower O₂ concentrations compared with normal tissues (1–6). Lymphocytes are unique, in that they circulate in the body via both blood circulation and lymphatics and are therefore exposed to variable O₂ concentrations. Upon encountering cognate antigens, lymphocytes are activated by antigen-presenting cells (APCs) in secondary lymphoid tissues, such as lymph nodes and spleen, where the oxygen tension is generally much lower than in the atmosphere or in the blood (7). After activation, lymphocytes leave the secondary lymphoid organs and enter the bloodstream where O₂ concentration is higher than in the secondary lymphoid organs. Lymphocytes are thus exposed to drastic changes in O₂ concentrations during antigen-induced activation and differentiation. However, the effect of changes in O₂ concentrations on T-cell activation and differentiation is not clear.

In hypoxic conditions, cellular metabolism and molecular signaling pathways are markedly affected and cells produce less ATP than under aerobic conditions since glycolysis is the only pathway generating ATP. Under aerobic conditions, however, cells produce ATP through both the glycolysis and the tricarboxylic acid (TCA) cycle. Hypoxia thus affects various cellular functions. For example, macrophages up-regulate the expression of CD11b and CD18 molecules (αM-integrin and β₂-integrin, respectively) under hypoxia, which promotes the migration of macrophages through the vascular endothelium into inflamed hypoxic tissues (8). On the other hand, the phagocytic activity of macrophages is decreased under hypoxic conditions (9) with associated low production of
reactive-oxygen species (ROS) and impaired lysosomal acidification (10) due to the insufficiency of O2. Neutrophil-mediated lung inflammation is also regulated by O2 tension in mice in vivo (11). The exposure of mice to hypoxic conditions resulted in the reduction of neutrophil-mediated pro-inflammatory responses and protected lung tissues from inflammation-mediated injuries.

CD4+ T<sub>r</sub> cells play a central role in adaptive immune responses. Upon recognition of their specific antigen in the secondary lymphoid organs, naive CD4<sup>+</sup> T cells differentiate into T<sub>1</sub>, T<sub>2</sub>, T<sub>17</sub> or regulatory T (Treg) cells (12, 13). These distinct types of T<sub>r</sub> subsets orchestrate host defense responses against various pathogens. Dysregulation of T<sub>r</sub>-cell responses results in the development of inflammatory and autoimmune diseases (14, 15). Because T<sub>r</sub> cells go through differentiation under hypoxic conditions in the secondary lymphoid tissues, it is expected that the effector function of T<sub>r</sub> cells will be affected by changes in O2 concentrations. Previous studies have examined the effect of hypoxia on T-cell functions in vitro. For example, exposure to hypoxia (1% O2) results in the prolonged impairment of cytokine expression (16), a shift toward T<sub>1</sub>2 responses and inhibition of T<sub>1</sub>1 responses (17). However, the effect of changes in O2 concentration on the differentiation of T<sub>r</sub> subsets has not been fully elucidated.

A key regulator of the cellular response to hypoxia is a transcription factor, hypoxia-inducible factor 1 (HIF-1) (18). The predominant form of HIF-1 is a heterodimer consisting of HIF-1α and HIF-1β subunits, both of which are members of the basic helix–loop–helix family of transcription factors. Although HIF-1β is constitutively expressed, the expression of the HIF-1α subunit is regulated by O2 concentrations. The HIF-1α protein is degraded in normoxic conditions but stabilized under hypoxic conditions. The stability of HIF-1α is regulated by a family of oxygen- and iron-dependent prolyl hydroxylases, whose activities direct the rapid degradation of HIF-1α by the ubiquitin-proteasome pathway. This process is dependent upon the von Hippel–Lindau tumor suppressor protein (vHL). Under hypoxia, prolyl hydroxylase activity is inhibited, resulting in the stabilization of HIF-1α. HIF-1α then translocates to the nucleus, where it binds the constitutively expressed HIF-1β. The resulting HIF-1 complex regulates the expression of genes involved in angiogenesis, oxygen transport, glucose metabolism and vascular tone (19) as well as the function of CTL (20).

Previous studies have shown the importance of mammalian target of rapamycin (mTOR) in immune cells. mTOR, a highly conserved serine/threonine kinase, controls a variety of cellular functions, such as cell growth, proliferation and survival (21). mTOR is activated downstream of various signaling pathways, one of which is the PI3K-Akt pathway. We and others have previously reported that molecules downstream of PI3K including mammalian target of rapamycin complex 1 (mTORC1) and GSK3β regulate the production of IL-12 and IL-10 in dendritic cells (DCs) (22–24). mTORC1 is also involved in the differentiation of T<sub>r</sub> cells (25, 26).

In this study, we demonstrate that T<sub>r</sub>17 differentiation is promoted under physiological O2 conditions. During the priming of T<sub>r</sub>17 cells under 5% O2, the expression of HIF-1α is increased via activation of mTORC1; the resulting HIF-1α promotes positive feedback activation of mTORC1, thereby leading to accelerated T<sub>r</sub>17-cell differentiation.

**Methods**

**Mice**

All animal experimental procedures conformed to the guidelines for animal experimentation administered by the Animal Care and Use Committee of Keio University. C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). T cell-specific HIF-1α-deficient mice, namely Lck-cre;Hif-1α<sup>fl/fl</sup> (HIF-1α-CKO) were generated by crossing Lck-cre and HIF-1α<sup>fl/fl</sup> mice purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Hif-1α<sup>fl/fl</sup> mice were used as a control. Mx-1<sup>cre</sup>;vHl<sup>fl/fl</sup> mice (vHL-CKO) were described previously (27). In brief, vHL<sup>fl/fl</sup> mice were mated to IFN-inducible Mx-1<sup>cre</sup> transgenic mice to generate vHL-CKO mice. Bone marrow (BM) mononuclear cells from vHL-CKO were transplanted into lethally irradiated C57BL/6-Ly5.1 mice. Cre expression in the transplanted BM was induced by intra-peritoneal injection of 400 μg poly IC on three alternate days. Splenocytes were then collected and naive T cells prepared. vHL<sup>fl/fl</sup> BM were used as a control. All mice were maintained in our SPF animal facilities and 6- to 10-week-old mice were used for all experiments unless otherwise stated.

**Reagents and antibodies**

Recombinant mouse IL-6 and human transforming growth factor (TGF)-β1 were purchased from R&D Systems (Minneapolis, MN, USA). Recombinant mouse IL-12 was obtained from PeproTech (Rocky Hill, NJ, USA). PE-conjugated anti-mouse IL-17A (TC11-18H10) and FITC-conjugated anti-mouse IFN-γ (XMG-1-2) antibodies were purchased from BD Bioscience (San Jose, CA, USA). Anti-p70<sup>S6K</sup> (sc-230), anti-β-actin (sc-7778) and anti-HIF-1α (sc-10790) were purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-phospho-p70<sup>S6K</sup> (Thr389) was purchased from Cell Signaling Technology (Danvers, MA, USA). Brefeldin A was purchased from eBioscience (San Diego, CA, USA). Rapamycin, phorbol myristate acetate (PMA) and ionomycin were purchased from Sigma–Aldrich (St Louis, MO, USA). Mice were used as a control.

**Isolation of naive CD4<sup>+</sup> T cells**

CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>high</sup> (naive CD4<sup>+</sup> T cells) were purified (purity: >95%) from splenocytes using a CD4<sup>+</sup>CD26<sup>+</sup> T cell isolation kit II and auto-MACS (Miltenyi Biotec) according to the manufacturer’s protocol.

**Cell culture**

RPMI1640 (Sigma) supplemented with 10% FCS (Equitech-Bio, Kerrville, TX, USA), 50 μM 2-mercaptoethanol (Gibco, Gaithersburg, MD, USA), 100 μM penicillin (Gibco), 100 μg ml<sup>−1</sup> streptomycin (Gibco), 1 mM sodium pyruvate (Gibco), 1× non-essential amino acids (Sigma) and 10 mM HEPES (Sigma) was used as a complete culture medium. MACS-purified naive CD4<sup>+</sup> T cells were cultured in 96-well plates (2.0 × 10<sup>5</sup> cells per well) and stimulated with immobilized anti-CD3e antibody (plates coated with 5 μg ml<sup>−1</sup>
anti-CD3ε antibody (145-2C11) in 50 µl PBS] and 1 µg ml⁻¹ soluble anti-CD28 antibody (37.51). Culture medium was supplemented with 10 ng ml⁻¹ IL-12 for Tₘ1-cell differentiation, 2 ng ml⁻¹ TGF-β for iTreg differentiation or 30 ng ml⁻¹ IL-6 and 3 ng ml⁻¹ TGF-β for Tₘ17-cell differentiation. For normoxia culture conditions, cells were maintained in a humidified incubator (Steri-cycle) containing 21% O₂, 5% CO₂ and 74% N₂ at 37°C. For hypoxia culture condition, cells were incubated in a humidified hypoxic incubator (ASTEC or BioSpherix) flushed with a gas mixture of 1–10% O₂, 5% CO₂ and 85–94% N₂ at 37°C for 36 h followed by normoxia for 24 h. For normoxia–hypoxia culture conditions, cells were cultured in a normoxic incubator containing 21% O₂, 5% CO₂ and 74% N₂ at 37°C for 36 h followed by culture in a hypoxic incubator containing 5% O₂, 5% CO₂ and 90% N₂ at 37°C for 24 h.

Intracellular staining of cytokines and transcription factors

Intracellular cytokine staining was performed using Intra Prep (Beckman Coulter, Brea, CA, USA) according to the manufacturer’s protocol. Tₘ cells were collected and re-stimulated with 50 ng ml⁻¹ PMA and 1 µg ml⁻¹ ionomycin for 2 h, followed by incubation with 3 µg ml⁻¹ Brefeldin A for an additional 2 h. A PE anti-mouse/rat Foxp3 staining kit (eBioscience) was used for the intracellular staining of Foxp3. Flow cytometry was performed on a FACSCalibur (BD Biosciences) and data were analyzed using Flowjo Software (Tree Star, Ashland, OR, USA).

Enzyme-linked immunosorbent assay

The concentration of IL-17A in culture supernatants was quantified by Quantikine Colorimetric Sandwich ELISAs (R&D Systems) according to the manufacturer’s instructions.

SDS–PAGE and western blotting

For detecting p70S6K, phospho-p70S6K and β-actin, cells were collected, lysed in a lysis buffer solution (1% Triton X-100, 20 mM Tris, pH 7.5, 12.5 mM β-glycerophosphate, 2 mM EDTA, 10 mM sodium fluoride, 1 mM benzamidine, 10 µg ml⁻¹ apro tinin, 50 µM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate and 2 mM dithiothreitol), applied to SDS–PAGE and transferred to polyvinylidifluoride membranes. An ECL Advance Western Blotting Detection Kit (GE Healthcare, Waukesha, WI, USA) was used for the detection of chemiluminescence. For detecting HIF-1α, cells were collected and lysed in a lysis buffer solution (1% Triton X-100, 20 mM Tris, pH 7.5, 12.5 mM β-glycerophosphate, 2 mM EDTA, 10 mM sodium fluoride, 1 mM benzamidine, 10 µg ml⁻¹ apro tinin, 50 µM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 10 µM MG132 and 2 mM dithiothreitol) and VECTASTAIN (Vector Laboratories, Burlingame, CA, USA) used for the detection. An LAS-3000 imaging system (Fujifilm, Tokyo, Japan) was used to quantitate digital images. The data were quantitated by ImageJ.

Quantitative real-time PCR

Total RNA was extracted using Nucleospin RNA II (Macherey-Nagel, Düren, Germany). cDNA was reverse transcribed using a High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer’s protocol. A CFX96 Real Time PCR System, C1000 Thermal Cycler and Sso Fast EvaGreen supermix (all from Bio-Rad) were used to evaluate gene expression. The expression level of each gene was normalized to 18s rRNA expression. The primer for Rorc (28) was previously described. The following primer sequences were used to detect other mRNAs: Il17a, sense primer, 5’-GACCTGATAGATATCCCTCTG-3’; anti-sense primer, 5’-CAGAATTCATGTGGTGTAGCTCCAG-3’; 18s rRNA, sense primer, 5’-CCGCCGTAGAGTGGAAATCTT-3’; anti-sense primer, 5’-CAGACCTCGACCATTTGTTCT-3’; Hif1α, sense primer, 5’-CACCTCCACAAATGTGACCT-3’; anti-sense primer, 5’-CCACACTGAGGTTGTTACT-3’; Vegf sense primer, 5’-CCACGTCAAGAGCACAATCA-3’; anti-sense primer, 5’-TCATTCTCTCTATGTGCTGGCTTT-3’; Glut1 sense primer, 5’-CATCCTTATTGCCAGTGTTT-3’; anti-sense primer, 5’-GAAGACGACACTGACGAC-3’.

Lentiviral transduction

The mouse Hif-1α was sub-cloned into CSII-EF-MCS-IRE2-Venus. The following constructs were kindly provided by Dr H. Miyoshi (RIKEN, Tsukuba, Japan): CSII-EF-MCS-IRE2-Venus, a self-inactivating lentiviral construct; pCAG-HIV/gp and pCMV-VSVG-RSV-REV, packaging constructs. This lentiviral system was designed to express a desired gene under the direction of the elongation factor-1 promoter along with internal ribosomal entry site (IRE5)-driven Venus, a derivative of GFP, as a marker for monitoring the infection efficiency. For the generation of lentiviral vectors, 293T cells were transfected with CSII-EF-MCS-IRE2-Venus with or without Hif-1α, pCAG-HIV/gp and pCMV-VSVG-Rev using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 2 days, culture supernatants were passed through a 0.45-µm filter, condensed to 0.5% volume and used for gene transduction. For viral transduction, freshly sorted naive CD4+ T cells were stimulated on day 0 in a 24-well plate with immobilized anti-CD3ε antibody and soluble anti-CD28 antibody as described above. On the same day, fresh lentiviral supernatant was added and cells were spin infected at 2000 r.p.m. for 2 h at room temperature. On day 1, cells were washed and cultured again under Th17-inducing conditions. Cells were harvested on day 3 and analyzed by intracellular cytokine staining and flow cytometry. The efficiency of viral infection examined by GFP expression was ~0.5%

Statistics

Mean values and standard errors of the mean values were calculated from at least three independent experiments. P values indicated in each graph were calculated using a two-tailed unpaired Student’s t-test.

Results

Reoxygenation accelerates the differentiation of Tₘ17 cells

We first examined the differentiation of Tₘ1_, iTreg or Tₘ17 cells primed under hypoxic (1 or 5% O₂) conditions for 36 h followed by cultivation under normoxia for 24 h. The differentiation of all Th subsets was severely impaired under 1% O₂ (Fig. 1A–C), a condition which was associated with strong
Fig. 1. Reoxygenation accelerates the differentiation of Th17 cells. (A–C) Naive CD4^+ T cells isolated from C57BL/6 mice were activated with anti-CD3 and anti-CD28 using Th1-, iTreg- and Th17-inducing conditions under normoxia, 1 or 5% O_2 for 36 h followed by normoxia for 24 h. Intracellular cytokine content was determined by flow cytometry after the 60-h culture. Cells were cultured in triplicate and the experiment was repeated three times with similar results (mean ± SD, **P < 0.01, n.s = not significant). (D and F) mRNA were collected from Th17 cells primed under normoxia (open boxes) or reoxygenation (closed boxes) for the indicated times. Expression levels of IL-17A (Il17a in figure) and Rorc (Rorc in figure) mRNAs were analyzed by real-time PCR and normalized to 18s rRNA. The experiments were repeated three times with similar results (mean ± SD, **P < 0.01, *P < 0.05). (E) The culture supernatants were collected from Th17 cells primed under normoxia (open boxes) or reoxygenation (closed boxes) for the indicated times. The secretion of IL-17A was measured by ELISA. The experiments were repeated three times with similar results (mean ± SD, **P < 0.01). (G) Naive CD4^+ T cells were cultured under normoxia for 60 h (normoxia), primed under normoxia for 36 h followed by 5% O_2 for 24 h (normoxia–hypoxia) or primed under 5% O_2 for 36 h followed by normoxia for 24 h (reoxygenation) and the percentage of IL-17A-positive cells determined by flow cytometry (n = 3). (H) Th17 cells were differentiated under the indicated O_2 concentrations for 36 h followed by reoxygenation for 24 h and intracellular cytokine content was determined by flow cytometry (n = 3). (I) The number of Th17 cells present after cultures were primed under normoxia (open diamonds), under 5% O_2 (black boxes) or 1% O_2 (black triangles) for 36 h followed by reoxygenation for 24 h were counted at 36 and 60 h. The experiments were repeated with similar results (mean ± SD, **P < 0.01, n = 3).
inhibition of cell growth (data not shown). We noted that the differentiation of Th17, but not Th1 or iTreg, was significantly accelerated by priming under 5% O2 compared with 21% followed by reoxygenation (Fig. 1A–C). Under these conditions, it was difficult to detect IL-4+ cells because a 60-h incubation was not sufficient for full Th2 differentiation (data not shown). Consistent with this observation, the expression level of IL-17A mRNA (Fig. 1D) as well as the amount of IL-17A protein secreted (Fig. 1E) were higher under reoxygenation conditions compared with induction of Th17 cells with normoxia throughout the incubation time. Furthermore, the expression level of mRNA encoding a critical transcription factor for Th17-cell differentiation, namely RORc, was slightly higher under the reoxygenation condition compared with the constant normoxic condition (Fig. 1F).

The differentiation of Th17 cells was greatly suppressed when cells were primed under normoxia for 36 h and transferred to hypoxia (normoxia–hypoxia condition) (Fig. 1G). We also examined the optimal O2 concentration for the differentiation of Th17 cells and found that priming under 3–5% O2 for 36 h significantly increased the differentiation of Th17 cells (Fig. 1H). It should be noted that the cell numbers after priming under 5% O2 were similar to those after priming under normoxic condition, whereas the viability of cells primed under 1% O2 was greatly reduced (Fig. 1I).

mTORC1 accelerates the differentiation of Th17 cells primed under 5% O2

The differentiation of Th17 cells involves the activation of mTORC1 (25, 26). To investigate whether mTORC1 is involved in the acceleration of Th17 differentiation under hypoxic conditions, we first examined whether mTORC1 is activated during Th17 differentiation under hypoxic conditions. As shown in Figure 2(A), we observed that the phosphorylation of p70S6K, a known target of mTORC1, was induced in Th17 cells cultured in 5% O2. The increase in p70S6K phosphorylation was maintained even after cells were exposed to normoxia for 2 h compared with cells that were maintained under constant normoxia (Fig. 2A). To test whether mTORC1 activity is important for the acceleration of Th17 differentiation under hypoxia, we added rapamycin, an inhibitor of mTORC1, to the culture media. Figure 2(B) shows that Th17-cell differentiation was reduced and the acceleration of Th17-cell differentiation by reoxygenation...
was completely inhibited by rapamycin, demonstrating the importance of mTORC1 in both the induction and the acceleration of Th17-cell differentiation. We next investigated whether the activation of mTORC1 in Th17 cells primed under 5% O2 was regulated by PI3K–Akt axis. The acceleration of Th17 cells primed under 5% O2 was observed in the presence of IC87114, a specific inhibitor for p110α, a leukocyte-specific catalytic subunit of the class IA PI3K or LY294002, a pan PI3K inhibitor (Fig. 2C, left panels), and the phosphorylation of p70^S6K was increased in spite of the adding these PI3K inhibitors (Fig. 2C, right panels), indicating that activation of mTORC1 with hypoxia is independent of the PI3K–Akt axis. It should be noted that Th17 differentiation was generally suppressed by IC87114 or LY294002 (Fig. 2C, left panels) even though the acceleration of Th17 differentiation was not blocked by those inhibitors. Taken together, it is likely that Th17 differentiation is positively regulated by mTORC1 downstream of PI3K–Akt axis. In addition, mTORC1 activated by a hypoxia-dependent and PI3K–Akt-independent pathway accelerates Th17-cell differentiation under reoxygenation conditions.

**Hypoxia-induced activation of mTORC1 controls the expression of HIF-1α in Th17 cells**

HIF-1α is a key factor in cellular adaptation to hypoxia. Recent studies have shown that, in addition to hypoxia, HIF-1α is induced by various pathways including cytokine receptor signaling, the PI3K–Akt pathway (29) and mTORC1 (30). We observed that the expression level of HIF-1α in Th17 cells, but not Th1 cells, induced under 5% O2 for 36 h was increased compared with in cells under normoxia, and the observed increase during Th17 differentiation was canceled by treatment with rapamycin (Fig. 3A). The elevation of HIF-1α expression in cells cultured under 5% O2 and its suppression by rapamycin were also observed at the mRNA level (Fig. 3B). In addition, the expression of VEGF and GLUT1 mRNAs, target genes of the HIF-1 complex, was also inhibited by rapamycin treatment (Fig. 3B). These data collectively show that the expression of HIF-1α during Th17 differentiation under hypoxia is regulated by the activation of mTORC1.

**HIF-1α modulates the differentiation of Th17 cells via activation of mTORC1**

Shi et al. recently reported that the differentiation of Th17 cells was impaired in the absence of HIF-1α and that this was associated with increased Foxp3 expression (31). Foxp3 antagonizes RORγt function and Th17 differentiation (32). In our experimental system, the percentages of Foxp3+ cells in Th17 cells cultured under normoxia and under reoxygenation conditions were comparable (Fig. 4A), suggesting that the Foxp3 level is not involved in the acceleration of Th17 differentiation by reoxygenation. To investigate the role of HIF-1α in the acceleration of Th17 differentiation by reoxygenation, we differentiated naive CD4+ T cells from T cell-specific HIF-1α-conditional knockout (HIF-1α-CKO) or control mice into Th17 cells primed under normoxia or 5% O2. After 36 h of culture, we analyzed the activation of mTORC1 as examined by the phosphorylation of p70^S6K. In control T cells, the phosphorylation of p70^S6K was promoted under 5% O2 compared with normoxia. By contrast, in HIF-1α-CKO T cells, the phosphorylation of p70^S6K was unaffected by the change of O2 concentration (Fig. 4B), suggesting that the activation of mTORC1 under 5% O2 promotes a positive feedback loop between HIF-1α and mTORC1.

Furthermore, the differentiation of Th17 cells primed under 5% O2 was not accelerated in the absence of HIF-1α (Fig. 4C). In contrast to the HIF-1α-CKO T cells, the acceleration of differentiation of Th17 cells under reoxygenation conditions was significantly greater in Th cells lacking vHL compared with control T cells (Fig. 4D). Since HIF-1α is stably expressed in the absence of vHL, these results confirm that HIF-1α is involved in the acceleration of Th17 differentiation by hypoxia. To further confirm the role of HIF-1α in Th17 cells, we transfected and over-expressed HIF-1α in naive CD4+ T cells and then examined their differentiation into Th17 cells. The percentage of IL-17A+ CD4+ T cells was increased in the population transfected HIF-1α (GFP+) (47%) compared with those not transfected HIF-1α (GFP-) (23%) (Fig. 4E). Taken together, HIF-1α induced by the activation of mTORC1 under 5% O2 promoted positive feedback activation of mTORC1, leading to the acceleration of Th17 cells differentiation.

**Discussion**

Tissue culture technique has enabled us to investigate in vitro the cellular functions reflected by the role of various cells in vivo. However, *in vitro* systems do not exactly mimic physiological conditions and artificial environments often profoundly influence the experimental results (33, 34). Many researchers have studied the conditions in which tissue culture systems conform to physiological conditions (35). There is, however, the caveat that most experimental systems have neglected to consider the O2 concentrations that exert a great deal of influence on cellular functions.

We have established in this study a novel model of analyzing Th1 differentiation, which reflects the changes of O2 concentration found *in vivo*. As shown here, the differentiation of Th17 cells is promoted by priming under a low O2 concentration (5% O2) followed by normoxic condition via the positive feedback activation of mTORC1 and HIF-1α. Although most studies on Th1-cell differentiation have been carried out in CO2 incubators which contain 5% CO2 and 21% O2, there were some reports in which Th1 differentiation was examined under hypoxia. For example, the suppressive activities of Tregs and Foxp3+CD4+CD25+ cells were enhanced under 1% O2, which mimicked conditions at inflammatory sites (36). We focused in this study on Th17 differentiation in the secondary lymphoid organs but not in inflammatory sites. Nevertheless, we noted that cell viability was greatly reduced when Tregs were induced from naive splenic T cells under 1% O2 followed by reoxygenation. To mimic the physiological conditions where naive CD4+ T cells are activated in the secondary lymphoid organs and then released into the bloodstream, we primed naive CD4+ T cells under 5% O2, a concentration of O2 found in the lymph nodes or spleen (7), and reoxygenated under normoxia which mimicked the O2 concentration in blood.
We primed naive CD4+ T cells for 36 h because this time point matched the time point when the proliferation of naive T cells is induced in response to antigen in vivo (37). Under these conditions, Th17 differentiation, but not Th1 or iTreg differentiation, was accelerated by reoxygenation. Our results are consistent with reports that local or systemic hypoxia might contribute to the up-regulation of T h17 differentiation and IL-17A expression in PBMC derived from severe ischemic stroke patients during the chronic stage (38). It was also reported that bone marrow-derived DCs (BMDCs) generated under hypoxia–reoxygenation conditions induced greater Th1, Th17 cell differentiation, which was associated with the production of higher levels of IFN-γ and IL-6 when compared with BMDCs generated under normoxia (39). Taken together, hypoxia–reoxygenation conditions tend to induce pro-inflammatory responses, and the Th17 response, in particular.

It is known that ROS are produced in mitochondria under conditions of O2 stress and regulate a variety of hypoxic responses. Indeed, we observed ROS production during Th17 culture under 5% O2 for 36 h (data not shown), raising the possibility that ROS is involved in the acceleration of Th17 differentiation. However, the acceleration of Th17 differentiation primed under 5% O2 was not inhibited by N-acetyl-L-cysteine, a general ROS inhibitor (data not shown). These data suggest that ROS does not play an important role in the acceleration of Th17-cell differentiation.

Recent studies have reported that HIF-1α, a key transcription factor under hypoxic conditions, regulates the differentiation of Th17 cells (31). IL-6 and mTOR strongly induce HIF-1α expression during Th17 differentiation in comparison with Th1, Th2 or Treg cells. T cell-specific HIF-1α deletion impairs the differentiation of Th17 cells, which is associated with...
a decrease in IL-23R expression and an increase of Foxp3 expression. The induction of Foxp3+ cells was up-regulated under T\textsubscript{n}17-promoting conditions in HIF-1\textalpha-CKO T cells compared with control T cells as demonstrated by Shi et al. (31) (data not shown). However, priming under hypoxia did not affect the expression of Foxp3 in either HIF-1\textalpha-CKO or control
is activated by another environmental factor yet to be identified in media containing low concentrations of glucose or physiological concentrations of O2 (5% O2) in the differentiation of Th17 cells primed under 5% O2 was also promoted compared with that in which HIF-1α was over-expressed by using lentiviral transfection and HIF-1α was stabilized in an mTORC1-dependent manner. Furthermore, the activation of mTORC1 was not observed in HIF-1α-deficient T cells and Th17 differentiation was not promoted by reoxygenation in the absence of HIF-1α. Consistent with this observation, Th17 differentiation in vHL-deficient T cells in which HIF-1α accumulates was further accelerated by reoxygenation. Moreover, Th17 differentiation in which HIF-1α was over-expressed by using lentiviral transfection was promoted compared with that in which HIF-1α was not over-expressed. These data collectively indicate that hypoxia activates mTORC1, which enhances HIF-1α expression and HIF-1α promotes positive feedback to further activate mTORC1. It is unknown at the moment whether mTORC1 is directly activated by HIF-1α.

In conclusion, the differentiation of Th17 cells primed under physiological concentrations of O2 (5% O2) in the secondary lymphoid tissues is accelerated by reoxygenation. Such accelerated Th17-cell differentiation is regulated by a positive feedback loop between mTORC1 and HIF-1α. These findings suggest that control of the HIF-1 complex can be exploited for the immunotherapy of autoimmune diseases induced by Th17 cells.

Acknowledgements

We thank A. Minowa, Y. Hirata and Y. Baba for technical assistance; K. Takei and K. Hidaka for animal care.

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Disclosure

S.K. is a consultant for Medical and Biological Laboratories, Co. Ltd. The authors otherwise have no financial conflicts of interest.

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Funding

This work was supported in part by a grant-in-aid for Young Scientist (B) (21790476 to S.N.) from the Japan Society for the Promotion of Science and a Scientific Frontier Research Grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan.
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