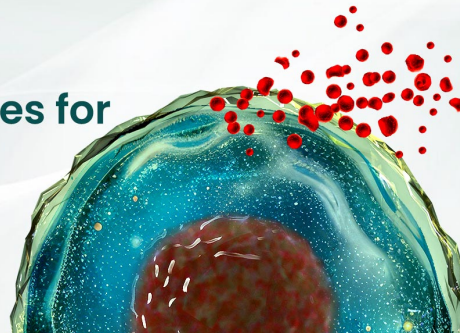




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Cutting Edge: Hypoxia-Inducible Factor 1 Negatively Regulates Th1 Function

Hussein Shehade, Valérie Acolty, Muriel Moser,¹ and Guillaume Oldenhove¹

Tissue hypoxia can occur in physiological and pathological conditions. When O₂ availability decreases, the transcription factor hypoxia-inducible factor (HIF)-1 α is stabilized and regulates cellular adaptation to hypoxia. The objective of this study was to test whether HIF-1 α regulates T cell fate and to define the molecular mechanisms of this control. Our data demonstrate that Th1 cells lose their capacity to produce IFN- γ when cultured under hypoxia. HIF-1 α ^{-/-} Th1 cells were insensitive to hypoxia, underlining a critical role for HIF-1 α . Our results point to a role for IL-10, as suggested by the increased IL-10 expression at low O₂ levels and the unchanged IFN- γ production by IL-10-deficient Th1 cells stimulated in hypoxic conditions. Accordingly, STAT3 phosphorylation is increased in Th1 cells under hypoxia, leading to enhanced HIF-1 α transcription, which, in turn, may inhibit suppressor of cytokine signaling 3 transcription. This positive-feedback loop reinforces STAT3 activation and downregulates Th1 responses that may cause collateral damage to the host. *The Journal of Immunology*, 2015, 195: 1372–1376.

There is increasing evidence that metabolic cues have a profound effect on the efficiency of innate and adaptive immune responses. Despite appropriate activation in lymphoid organs, the effector function of T lymphocytes may be regulated in the tissue microenvironment, eventually impeding the clearance of the Ag/pathogen (1). A typical example is hypoxia, which is a common feature of solid tumors and inflammatory sites (2). Hypoxic conditions in tumors result from irregular distribution of vessels in growing tumors, whereas the source of hypoxia during inflammation is edema, vasculitis, vasoconstriction (limiting oxygen delivery), and recruitment of polymorphonuclear cells (high oxygen consumers) (3). In addition, in the steady-state, the gut displays a complex oxygenation profile, with a high oxygenation level at the richly vascularized subepithelium that decreases gradually to the almost anoxic lumen (2).

The major oxygen sensor is hypoxia-inducible factor (HIF)-1 α , which is degraded under normoxia and stabilized under

hypoxia (4). The transcription factor HIF-1 (composed of the regulated HIF-1 α and the constitutive HIF-1 β subunits) functions as a master regulator of oxygen homeostasis and mediates the metabolic switch from oxidative phosphorylation to glycolysis in response to hypoxia (reviewed in Ref. 5). It is interesting to note that activated T cells rely on glycolysis to generate ATP (6, 7), an observation compatible with a role for HIF-1 in the regulation of T cell development and function (reviewed in Ref. 8).

The primary objective of our study was to examine the impact of hypoxia on cytokine production by activated Th subsets. Two recent reports clearly showed that HIF-1 promoted Th17 and attenuated regulatory T cell (Treg) development in normoxic conditions (9, 10). However, the effect of hypoxia on Th1 cell function remains elusive and, therefore, was the object of the current study.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were purchased from Harlan Nederland. C57BL/6 HIF-1 α ^{lox/lox} mice were provided by Dr. F. Bureau (Université de Liège), CD4 Cre mice were provided by Dr. G. Van Loo (Ghent University), STAT3^{lox/lox} mice were provided by Dr. S. Akira (Osaka University); IL-21R^{-/-} mice were provided by Dr. W.J. Leonard (National Institutes of Health); and IL-6^{-/-} mice and BALB/c IL-10^{-/-} mice were provided by Dr. V. Flamand (Institute for Medical Immunology). IFN- γ YFP and IL-10 GFP reporter mice were purchased from The Jackson Laboratory.

Cell purification and culture media

Splenic CD4⁺CD25⁻ T cells were obtained by negative selection, and CD11c⁺ cells were enriched from low-density spleen cells by positive selection, using an autoMACS (Miltenyi Biotec). The medium used was RPMI 1640 supplemented with 10% FCS and additives.

T cell differentiation

T cells were stimulated for 3 d in 24-well plates containing immobilized anti-CD3 and soluble anti-CD28 Abs in RPMI 1640 media supplemented with IL-12 and anti-IL-4 mAbs for Th1 differentiation; IL-2, TGF- β , anti-IFN- γ , and anti-IL-4 mAbs for Treg differentiation; or IL-6, TGF- β , anti-IFN- γ , and anti-IL-4 mAbs for Th17 differentiation. Polarized T cells were rested for 24 h and cultured in the same polarizing conditions for 3 d in 20% O₂ (conventional incubator) or in chambers containing 1% O₂ using an Anoxomat (Mart Microbiology).

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The online version of this article contains supplemental material.

Abbreviations used in this article: CA, constitutively activated; HA, hemagglutinin; HIF, hypoxia-inducible factor; KLH, keyhole limpet hemocyanin; ND, nondegradable; SOCS, suppressor of cytokine signaling; Treg, regulatory T cell; WT, wild-type.

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Phenotypic analysis

Cells were incubated with anti-FcγIII/IIR mAb and stained with fluorochrome-conjugated mAb against TCRβ-chain and CD4. A LIVE/DEAD kit (Molecular Probes) was used to exclude dead cells. Intracellular staining was performed using the Foxp3 staining set (eBioscience). T-bet staining was performed using anti-mouse/human T-bet mAbs (eBioscience). For pSTAT3 detection, cells were stained with a LIVE/DEAD kit, fixed for 20 min using 4% paraformaldehyde, permeabilized with 90% methanol for 30 min, and stained in PBS with mAbs against TCR-β, CD4, and pSTAT-3 (BD Biosciences).

In vitro restimulation and intracellular cytokine staining

For cytokine detection, cells were cultured at 1×10^6 cells/ml in a 96-well U-bottom plate and stimulated with 50 ng/ml PMA and 1 μg/ml ionomycin (Sigma) in the presence of brefeldin A (BD Biosciences). After 3 h, intracellular staining was performed according to the BD Cytotfix/Cytoperm kit protocol. Cells were stained with a LIVE/DEAD kit and with fluorochrome-conjugated Abs against TCRβ-chain, CD4, IFN-γ, IL-17, IL-10, or isotype controls (eBioscience).

Immunization and T cell readout ex vivo

A total of 5×10^5 keyhole limpet hemocyanin (KLH)-pulsed dendritic cells (incubated overnight with) or 10 μg KLH mixed with 10 μg LPS were injected into the footpads of mice. Draining lymph nodes were harvested 5 d later, and cells were plated in 96-well round-bottom plates in Click's medium (Sigma-Aldrich) supplemented with 0.5% heat-inactivated mouse serum and additives. Cells were restimulated with PMA/ionomycin in the presence of brefeldin A for 3 h and analyzed by flow cytometry for TCR-β, CD4, and IFN-γ expression. Alternatively, cells were restimulated with graded doses of KLH for 72 h, and IFN-γ content was measured in the supernatants by ELISA. Lymph node cells were stained for TCR-β, CD4, CD44, and CD62L expression before and after stimulation with KLH to assess activation.

Real-time PCR

RNA was extracted from cell lysates with TRIzol reagent. Quantitative PCR was performed using a StepOne Plus system (Applied Biosystems) with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific). Quantification (with ribosomal protein L32 as endogenous housekeeping gene) was done using standard curves. Levels of mRNA expression were normalized to ribosomal protein L32 mRNA.

Constructing hemagglutinin-tagged wild-type and nondegradable HIF-1α

cDNA sequence coding for murine HIF-1α was subcloned in a retroviral pMXs vector with an hemagglutinin (HA) tag added upstream (N-terminal domain) of the HIF-1α sequence. Proline 402 and 577 were mutated into alanine (Supplemental Fig. 2).

Retroviral transduction

Activated CD4⁺ T cells were spin infected with retrovirus-containing supernatant from Platinum-E retroviral packaging cells (kindly provided by Dr. T. Kitamura, University of Tokyo, Tokyo, Japan). The retroviral plasmid used was pMX-IRES-GFP, either empty or encoding constitutively activated (CA) STAT3 (kindly provided by Dr. Jacqueline F. Bromberg, Memorial Sloan-Kettering Cancer Center), or HA-tagged wild-type (WT) or mutant (nondegradable [ND]) HIF-1α.

Statistical analysis

Statistical analyses were performed using Prism6 (GraphPad Software). Statistical significance was determined with the Mann-Whitney *U* test for two-tailed data.

Results and Discussion

Decreased IFN-γ production by differentiated Th1 cells cultured in hypoxic conditions

To assess the effect of hypoxia on differentiated T cell subsets, CD4⁺ CD25⁻ T cells were purified from CD4 Cre HIF-1α^{fl/fl} or CD4 HIF-1α^{fl/fl} mice and activated with anti-CD3 and anti-CD28 mAbs for 3 d in vitro in Th1-, Th17-, or Treg-polarizing conditions. Cells were rested for 24 h and cultured at 20 or 1% O₂ in the presence of anti-CD3 mAb in the same

polarizing conditions. After 72 h, cells were harvested and analyzed by flow cytometry for cytokine production. The data in Fig. 1A (pool of eight experiments) and in Supplemental Fig. 1A indicate that the proportion of IFN-γ-producing cells was strongly decreased in hypoxic conditions compared with normoxic conditions (from 70 to 20%). In accordance with two recent reports (9, 10), the proportion of IL-17-producing cells was increased in hypoxia compared with normoxia (from 12.5 to 26.3%), whereas the proportion of Foxp3⁺ T cells decreased dramatically (from 91.6 to 28.7%). The changes in the proportion of Th1 cells (Fig. 1A), Th17 cells, and Tregs (Supplemental Fig. 1A) and the upregulation of GLUT-1 expression (Supplemental Fig. 1B, used as internal control), were abolished in HIF-1α-deficient cells. These observations complement previous studies showing that Th17 cells and Tregs were reciprocally regulated by HIF-1α in normoxia (9, 10) and further demonstrate that low-oxygen tension results in impaired function of activated Th1-type cells in an HIF-1α-dependent manner. The apparent discrepancy between our data and a previous report (11) showing that hypoxia increases Treg number and function could be due to different protocols, because we stimulated fully differentiated Foxp3⁺ Tregs in hypoxic conditions. Our data further show that the global proliferation of CD4⁺ T cells stimulated in 1% hypoxia also was diminished, as assessed by CFSE staining and cell counts (Supplemental Fig. 1C), indicating that hypoxia repressed Th1 proliferation and effector function.

Stabilization of HIF-1α results in decreased IFN-γ production

To better document the effect of HIF-1α on Th1 cells, we generated an ND form of HIF-1α. WT and ND HIF-1α were fused to an HA tag on their N-terminal domain and inserted in a pMX-IRES GFP retroviral vector. CD4⁺ T cells were cultured for 24 h in the presence of anti-CD3 and anti-CD28 mAbs in Th1-polarizing conditions, retrovirally transduced (in the same activating conditions) with either construct, and tested 3 d later for HA, T-bet, and IFN-γ expression by flow cytometry. The data in Fig. 1B clearly show that T cells transduced with the ND form of HIF-1α displayed higher expression of HA (due to stabilization of HA-HIF-1α fusion protein) and decreased levels of intracellular IFN-γ. The higher expression of HIF-1α correlated with increased mRNA coding for GLUT1 (Fig. 1C), as expected.

To further dissect the mechanism of suppression, we monitored the expression of mRNA coding for STAT4, a key pro-Th1 factor, in T cells expressing a WT or ND form of HIF-1α stimulated with anti-CD3 in normoxic conditions (Fig. 1D), as well as in T cells purified from CD4 Cre HIF-1α^{fl/fl} or CD4 HIF-1α^{fl/fl} cells activated in normoxic or hypoxic conditions (Fig. 1E). The data revealed an inverse correlation between HIF expression and STAT4 transcription. Collectively, these observations confirm the inhibitory role of stabilized HIF-1α on Th1 function in vitro and suggest that HIF-1 may inhibit STAT4 transcription.

STAT3 and suppressor of cytokine signaling 3 are involved in downregulation of Th1 function in hypoxia

STAT3 is a transcription factor with pleiotropic functions that appears to be a critical regulator of T cell proliferation and differentiation with opposite effects on Th17 cells and Th1

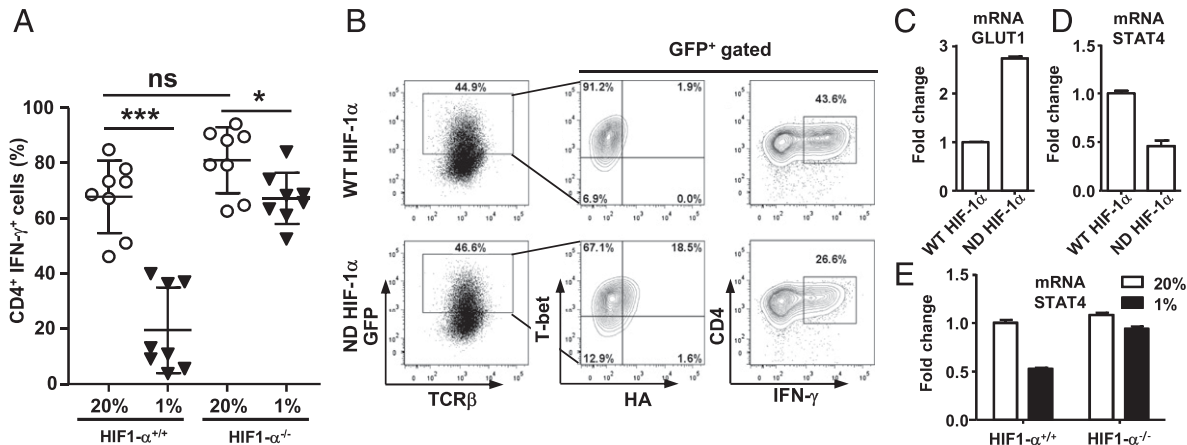


FIGURE 1. HIF stabilization results in impaired Th1 function. **(A)** CD4⁺ T cells were isolated from CD4 Cre HIF-1 α ^{fl/fl} or CD4 HIF-1 α ^{fl/fl} mice, activated under Th1-polarizing conditions, rested for 24 h, and cultured in 20 or 1% pO₂ for an additional 72 h. After a short restimulation with PMA/ionomycin in the presence of brefeldin A, cells were analyzed by flow cytometry for IFN- γ expression. Data are expressed as percentages of CD4⁺ IFN- γ ⁺ cells and are representative of eight independent experiments. **(B–D)** CD4⁺ T cells were stimulated in vitro with anti-CD3 and anti-CD28 mAbs and retrovirally transduced to express GFP and the HA-tagged WT or ND form of HIF-1 α . Cells were cultured under Th1-polarizing conditions for 72 h and restimulated with PMA/ionomycin in the presence of brefeldin A. **(B)** GFP⁺ transduced cells were analyzed by flow cytometry for T-bet, HA, and IFN- γ expression. Percentage of HA⁺ cells among CD4⁺ cells: 2.6 ± 1.8 versus 23.6 ± 13.1 , $p = 0.01$; percentage of IFN- γ ⁺ cells among CD4⁺ cells: 43.7 ± 9.8 versus 26.6 ± 6.9 , $p = 0.03$, for WT and ND HIF-1 α , respectively. **(C)** and **(D)** mRNA expression was measured in GFP⁺ sorted cells. **(E)** CD4⁺ T cells from CD4 Cre HIF-1 α ^{fl/fl} or CD4 HIF-1 α ^{fl/fl} mice were treated as in **(A)** and analyzed for STAT4 mRNA expression by qPCR. Bar graphs in **(C)–(E)** show the means of duplicate wells \pm SDs and are representative of at least four independent experiments. * $p < 0.05$, *** $p < 0.001$. ns, not significant.

cells/Tregs (12). The proportion of cells expressing phosphorylated STAT3 (Supplemental Fig. 2A) and the intensity of expression (Fig. 2A) were increased in differentiated Th1 cells cultured in hypoxic conditions compared with normoxic conditions, suggesting a role for STAT3 signaling in Th1 inhibition in hypoxia. Accordingly, the proportion of IFN- γ -producing cells was decreased among T cells expressing a CA form of STAT3 (Fig. 2B) and increased among STAT3-deficient T cells cultured in normoxia (92 versus 65%) and

mainly in hypoxia (76 versus 9%) compared with WT T cells (Fig. 2C). Because STAT3 activation was shown to be associated with increased HIF transcription (9, 13), we monitored the level of mRNA coding for HIF-1 α in cells expressing different levels of activated STAT3. The expression of HIF-1 α mRNA was lower in T cells from CD4 Cre STAT3^{fl/fl} mice compared with CD4 STAT3^{fl/fl} mice (Fig. 2D) and was higher in T cells expressing an active form of STAT3 (Fig. 2E). Finally, we examined the expression of mRNA coding

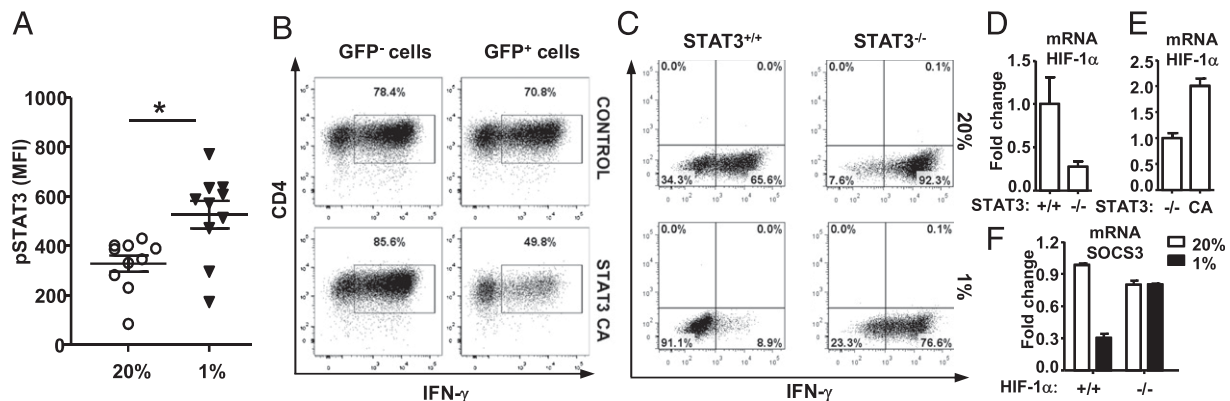


FIGURE 2. Role of STAT3 and SOCS3 in the regulation of Th1 function in hypoxia. **(A)** CD4⁺ T cells were isolated from C57BL/6 mice, cultured as described in Fig. 1A, and analyzed by flow cytometry for TCR- β , CD4, and pSTAT3 expression. Data represent the level of STAT3 phosphorylation (mean fluorescence intensity [MFI]) from 10 independent experiments, and horizontal lines represent median \pm interquartile range. **(B)** CD4⁺ T cells from C57BL/6 mice were activated with anti-CD3 and anti-CD28 mAbs and retrovirally transduced to express a CA form of STAT3 or the empty vector (control). Cells were cultured under Th1-polarizing conditions for 72 h and restimulated with PMA/ionomycin in the presence of brefeldin A. Nontransduced GFP⁻ and transduced GFP⁺ cells were analyzed by flow cytometry for IFN- γ secretion. The average percentage of IFN- γ ⁺ cells among CD4⁺ cells expressing the empty vector or STAT3 CA was 72.4 ± 4.5 and 48.5 ± 4.1 , respectively, $p = 0.028$. **(C)** CD4⁺ T cells from CD4 Cre STAT3^{fl/fl} or CD4 STAT3^{fl/fl} mice were treated as in Fig. 1A and analyzed by flow cytometry for IL-17 and IFN- γ expression. Data are representative of five independent experiments. Mean percentage of IFN- γ ⁺ cells among STAT3-competent CD4⁺ cells: 67.1 ± 10.7 versus 20.1 ± 13.9 , $p = 0.0079$; percentage of IFN- γ ⁺ cells among STAT3-deficient CD4⁺ cells: 89.1 ± 8.3 versus 70 ± 12.8 , $p = 0.055$, for 20 and 1% pO₂, respectively. Data are representative of five independent experiments. **(D)** and **(E)** CD4⁺ T cells from CD4 STAT3^{fl/fl} mice (STAT3 WT) or CD4 Cre STAT3^{fl/fl} mice transduced (STAT3 CA) or not (STAT3^{-/-}) with a CA form of STAT3 were activated for 72 h with anti-CD3 and anti-CD28 mAbs under Th1-polarizing conditions and analyzed for expression of mRNA coding for HIF-1 α . **(F)** CD4⁺ T cells from CD4 HIF-1 α ^{fl/fl} (HIF-1 α ^{+/+}) or CD4 Cre HIF-1 α ^{fl/fl} (HIF-1 α ^{-/-}) mice were treated as in Fig. 1 and analyzed for expression of mRNA coding for SOCS3. Bar graphs in **(D)–(F)** show mean (\pm SD) of duplicate wells; data are representative of three independent experiments. * $p < 0.05$.

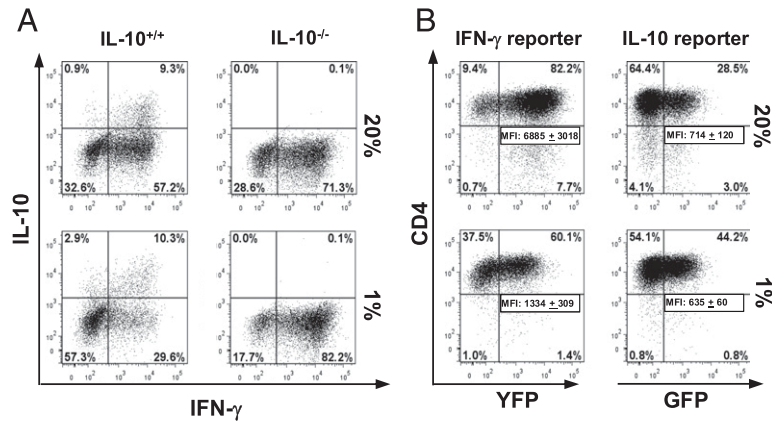


FIGURE 3. Role of IL-10. **(A)** CD4⁺ T cells from WT or IL-10^{-/-} mice were cultured as described in Fig. 1 and restimulated for 3 h with PMA/ionomycin in the presence of brefeldin A. Cells were stained for TCR-β, CD4, IFN-γ, and IL-10 and analyzed by flow cytometry. Dot plots illustrate IFN-γ and IL-10 production by CD4⁺ TCR-β⁺ gated cells. Data are representative of five independent experiments. Mean percentage of IFN-γ⁺ cells among IL-10-competent CD4⁺ cells: 62.3 ± 6.3 versus 23.2 ± 10.9, $p = 0.0079$; mean percentage of IFN-γ⁺ cells among IL-10-deficient CD4⁺ T cells: 65.3 ± 11.2 versus 71.3 ± 9.7, $p = 0.3$, in normoxia and hypoxia, respectively. **(B)** CD4⁺ T cells from IFN-γ YFP or IL-10 GFP reporter mice were cultured as described in Fig. 1. Cells were stained for TCR-β and CD4 and analyzed by flow cytometry. Dot plots illustrate IFN-γ and IL-10 expression by CD4⁺ cells. Data are representative of three independent experiments.

for suppressor of cytokine signaling (SOCS)3, a major negative feedback regulator of STAT3 function. SOCS3 mRNA expression was decreased in hypoxic conditions in HIF-1α-competent, but not HIF-1α-deficient, T cells (Fig. 2F). Collectively, these observations demonstrate that STAT3, probably via SOCS3, is involved in the negative regulation of IFN-γ production in hypoxic conditions.

IL-10 is required for downregulation of Th1 function in hypoxic conditions

To gain better insight into the activation of the SOCS3/STAT3 regulatory pathway, we tested whether the cytokines IL-6, IL-10, and IL-21, which are known to induce STAT3 activation (14), were involved in the Th1 negative feedback in hypoxia. Our data indicate that IFN-γ production by WT, IL-6-deficient (Supplemental Fig. 2B), and IL-21R-deficient (Supplemental Fig. 2C) CD4⁺ T lymphocytes was impaired under low O₂ pressure, whereas the capacity of T cells from IL-10^{-/-} mice to produce IFN-γ remained unaltered in hypoxia (Fig. 3A). A role for IL-10 in downregulation of Th1 function (as demonstrated using reporter mice) was further suggested by the increased IL-10 produced by Th1 cells activated in hypoxia (Fig. 3B).

HIF-1 negatively regulates Th1 priming in vivo

Our in vitro observations that HIF-1α is involved in downregulation of Th1 function in hypoxia prompted us to test its role in vivo. Using a model of Th1 priming with KLH-loaded dendritic cells, we found that the proportion of IFN-γ-expressing CD4⁺ T cells measured ex vivo (Supplemental Fig. 2D), as well as their activation (Supplemental Fig. 2E) and IFN-γ production (Fig. 4A) upon antigenic restimulation in culture, were significantly enhanced in CD4 Cre HIF-1α^{fl/fl} mice compared with CD4 HIF-1α^{fl/fl} mice, whereas the Ag-specific proliferation in culture remained unchanged (data not shown). A similar increase in IFN-γ secretion was observed in CD4 Cre HIF-1α^{fl/fl} mice primed with KLH and LPS as adjuvant (Fig. 4B).

Collectively, our data show that hypoxia increases IL-10 expression by differentiated Th1 cells, leading to STAT3 activation and decreased IFN-γ production. Activated STAT3

enhances HIF transcription, which, in turn, may inhibit SOCS3 transcription when stabilized in hypoxia, thereby favoring STAT3 activation and creating a positive feedback loop between HIF and STAT3. Whether HIF-1 or another sensor of hypoxia is involved in IL-10 upregulation remains to be determined.

Our observations are in agreement with previous reports showing decreased IFN-γ secretion by TCR-triggered T cells in vitro in hypoxic conditions (1, 15, 16), as well as more severe colonic inflammation (16) and increased antibacterial effect in HIF-1α-deficient mice in a murine model of sepsis (1).

The mechanism by which hypoxia restricts Th1 priming requires further investigation. A recent report (17) showed that decreasing O₂ tensions during in vitro restimulation of tumor-infiltrating CD8⁺ T cells decreased proliferation and induced IL-10 secretion in a dose-dependent manner, confirming a role for IL-10. Several mechanisms could be involved, including direct inhibition of Th1 activation by IL-10 in an

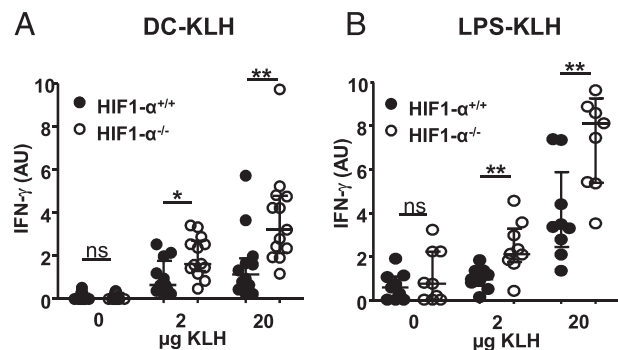


FIGURE 4. HIF-1 negatively regulates Th1 priming in vivo. KLH-pulsed dendritic cells from C57BL/6 mice **(A)** or KLH with LPS as adjuvant **(B)** was injected into the footpads of CD4 Cre HIF-1α^{fl/fl} or CD4 HIF-1α^{fl/fl} mice. Draining lymph nodes were harvested 5 d later and stimulated with graded doses of KLH, and IFN-γ was measured in supernatants by ELISA. Data are shown as individual mice and pooled from three (DC-KLH) or two (LPS-KLH) independent experiments. IFN-γ concentration is expressed in arbitrary units (AU) and normalized for each experiment to the mean value of IFN-γ produced (ranging from 0.2 to 5 ng/ml) by CD4 HIF-1α^{fl/fl} mice restimulated with 2 µg/ml KLH. Data are median ± interquartile range. * $p < 0.05$, ** $p < 0.01$. ns, not significant.

APC-independent manner (18, 19), inhibition of STAT4 expression by HIF-1, and repression of T-bet expression by STAT3 (12). In conclusion, we identified a molecular pathway linking hypoxia to STAT3 activation and decreased Th1 activation, which could represent a key regulator of Th1 effector function in inflamed peripheral tissues or in hypoxic sites in tumors.

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Disclosures

The authors have no financial conflicts of interest.

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