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Cutting Edge: Hypoxia-Inducible Factor 1α and Its Activation-Inducible Short Isoform I.1 Negatively Regulate Functions of CD4+ and CD8+ T Lymphocytes

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To evaluate the role of hypoxia-inducible factor 1α (HIF-1α) and its TCR activation-inducible short isoform I.1 in T cell functions, we genetically engineered unique mice with: 1) knockout of I.1 isoform of HIF-1α; 2) T cell-targeted HIF-1α knockdown; and 3) chimeric mice with HIF-1α gene deletion in T and B lymphocytes. In all three types of mice, the HIF-1α-deficient T lymphocytes, which were TCR-activated in vitro, produced more proinflammatory cytokines compared with HIF-1α-expressing control T cells. Surprisingly, deletion of the I.1 isoform, which represents <30% of total HIF-1α mRNA in activated T cells, was sufficient to markedly enhance TCR-triggered cytokine secretion. These data suggest that HIF-1α not only plays a critical role in oxygen homeostasis but also may serve as a negative regulator of T cells. The Journal of Immunology, 2006, 177: 4962–4965.

T cells are exposed to different levels of oxygen tension including very low (hypoxia) or the complete oxygen absence (anoxia) as they differentiate, mature, migrate, and function in different tissue microenvironments (1, 2). One of important mechanisms of adaptation of cells to the low oxygen tensions is based on activities of the hypoxia-inducible factor 1 (HIF-1) (reviewed in Ref. 3). HIF-1 was shown to be necessary for switching to glycolysis (4) and for promoting angiogenesis (5), thus ensuring the cells and tissues survival in hypoxic conditions. Since myeloid cells critically rely on glycolysis for their ATP production, it was shown that targeted deletion of HIF-1α in myeloid cells results in a dramatic decrease in inflammatory activity and survival of macrophages (6).

On the other hand, T cells are not expected to be dependent on HIF-1α for survival to the same extent as macrophages since T cells can produce ATP by both glycolysis and oxidative phosphorylation (7). Accordingly, we have proposed that changes in levels of HIF-1α may play not only the energy-providing but also a regulatory role in T cell functions in inflamed and hypoxic tissue microenvironments (2). This proposal was prompted by our earlier observations of increased inflammatory tissue damage in chimeric mice with complete HIF-1α deletion in cells of adaptive immune system (8). These observations suggested that HIF-1α in T cells may play the anti-inflammatory and tissue-protecting role by negatively regulating T cell functions (2, 9, 10). Our published hypothesis of the inhibitory role of HIF-1α in T cells (2) was recently supported by another group showing that HIF-1α overexpression leads to a decrease in TCR-triggered Ca2+ response (11). In addition, HIF-1α was shown to be involved in regulation of CD39, CD73, and A2B adenosine receptor molecules (12, 13), which are known to be involved in anti-inflammatory signaling by extracellular adenosine (14, 15).

HIF-1α expression in T lymphocytes can be induced both by hypoxia (16) and by non-hypoxic pathways, including TCR-triggered and PI3K-mediated pathways, resulting in mRNA up-regulation and protein stabilization (17–19). We previously demonstrated that T cell activation leads to “immediate early response gene” up-regulation of the alternative mRNA isoform of HIF-1α I.1 (20), which contains a different first exon and is expressed from self promoter in a immune tissue-specific manner (21, 22). Despite that I.1 isoform mRNA encodes protein 12-aa residues shorter than full-sized HIF-1α isoform I.2, it retains the DNA-binding and transcriptional activity (20, 23).

In this report, we describe studies of three new types of gene-altered mice that we developed to directly test the hypothesis whether HIF-1α is a negative regulator of T cells. We show that both known HIF-1α isoforms may function as negative regulators of T cell functions.

Materials and Methods
Generation of I.1-deficient mice
HIF-1α-I.1-deficient mice were developed using 129 mouse embryonic stem (ES) cells with targeted I.1 locus and C57BL/6 blastocysts to generate chimeric
offsprings, which were mated with C57BL/6 mice to generate F1 mice. An 0.5-kbp BamHI-HindIII fragment containing I.1 exon was replaced with “loxP-1.1 exon-Neo-loxP” cassette, which was subsequently excised by Cre. The targeting vector contained ~1.5-kbp XhoI-BamHI 5’ homology fragment, 1.5-kbp loxP-1.1 exon-loxP-neo-loxP cassette, 3-kbp HindIII-EcoRI 3’ homology fragment, and diphteria toxin A chain. DNA was digested with SacI/BamHI and analyzed by Southern blot using 32P-labeled EcoRI-SacI probe, which is located outside of the 3’ homology fragment of targeting vector. Properly targeted ES cell clones were transfected with Cre recombinase. ES cell clones with deleted I.1 were microinjected into C57BL/6 blastocysts to generate chimeric mice.

**Generation of mice with T cell-specific deletion of HIF-1α**

Mice with T cell-targeted deletion of HIF-1α gene (Lck-Cre+/HIF-1α-floxed) have been generated by breeding a homozygous HIF-1α-floxed mice (24) with the Lck-Cre transgenic mice (25). Mice that were HIF-1α-floxed and heterozygous for Lck-Cre had HIF-1α-deficient T cells, whereas control wild-type mice were their Lck-Cre-negative/HIF-1α-floxed siblings. Chimeric mice with lymphocyte-specific deletion of HIF-1α were described previously (8).

**Reagents**

All Abs were obtained from BD Biosciences except anti-HIF-1α mAb (Novus Biologicals). ELISA kits were purchased from R&D Systems. PMA and A23187 were from Sigma-Aldrich. Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM HEPES, and 50 μM 2-ME.

**RNA preparation and real-time PCR**

T lymphocytes were isolated from splenocytes, which were incubated with plate-bound anti-CD3/anti-CD28 mAb for 24 h, by auto-MACS separator (Miltenyi Biotec) using FITC-conjugated anti-CD4, anti-CD8 Abs and anti-FITC magnetic beads according to manufacturer’s protocol. RNA was extracted using RNA-STAT-60 (Tel-Test); cDNA was synthesized using 1 μg of total RNA using SuperScript first-strand synthesis kit (Invitrogen Life Technologies) with random hexamers. Real-time PCR was performed using SYBR Green master mix (Applied Biosystems) with L32 mRNA as an internal standard. Primers used: 1) CCCCGTCGCCCATCTCTC, CTGGGCTTCT TGCTTCACAGGAG; 2) CACCAGTCCGCGATGGA, TTGGACGT TCAGAATCATCTTTT; 3) AGCACAAAGAAAACCAAGCACAT, TTGACATTTGCGACAGAAT.

**Results and Discussion**

**Generation of mice with T cell-targeted deletion of HIF-1α isoforms**

We tested the hypothesis that HIF-1α serves as a negative regulator in activated T lymphocytes (2) using T cells from chimeric mice obtained using the Rag-2 blastocyst-complementation approach as described (8, 26). Then, we studied the role of HIF-1α by developing the HIF-1α isoform I.1-deficient mice. The I.1 exon was disrupted using the vector containing I.1 exon and neomycin resistance gene (neo) surrounded by loxP sites (Fig. 1A). After selection on G418, successfully targeted ES cell clones were found by Southern blot (Fig. 1B). The Neo gene and I.1 exon were subsequently removed via the transfection of vector expressing Cre recombinase (Fig. 1C). Chimeric mice were bred to produce HIF-1α-I.1+/– mice. To confirm that the mice indeed do not express I.1 isoform, mRNA from I.1+/– mice was analyzed by RT-PCR in comparison with wild-type control (Fig. 1D). For all experiments, I.1+/– and I.1+/+ siblings were used, which were produced by breeding of HIF-1α-I.1+/– heterozygous mice and screened by PCR (Fig. 1E).

Unlike the total knockout of HIF-1α (27), the disruption of I.1 isoform of HIF-1α did not result in embryonic death, and I.1–/– mice appeared normal. The RNase protection assay and real-time RT-PCR analysis of mRNA that are normally transcribed through HIF-1 activity (e.g., vascular endothelial growth factor, inducible NOS, Glut-1, lactate dehydrogenase, etc.) did not show significant changes due to the absence of I.1 isoform (data not shown). Moreover, thymocytes and T cells from immune organs of I.1–/– mice revealed no phenotypic differences from wild-type mice as checked by FACS analysis (data not shown).

**Enhanced cytokine production by HIF-1α-deficient CD4+ and CD8+ T lymphocytes**

First, we analyzed cytokine production by T cells that lack both isoforms of HIF-1α. We compared cytokine secretion by TCR-triggered HIF-1α-deficient CD8+ T cells (T cell line HID2) obtained from HIF-1α-deficient chimeras (8) and the matched HIF-1α-expressing control CD8+ T cells (line 129D2). We found that at both hypoxic (2% O2) and normoxic (20% O2) conditions, HIF-1α-deficient T cells secrete more IFN-γ than wild-type cells (Fig. 2A). In addition, we examined the per cell levels of IFN-γ in CD8+ T cells using intracellular staining. Flow cytometry analysis showed that HIF-1α-deficient CD8+ T lymphocytes produce more IFN-γ than wild-type control (Fig. 2B).

In addition, we showed higher IFN-γ secretion by activated splenocytes from Lck-Cre+/HIF-1α-floxed mice, which have T cell-specific deletion of HIF-1α (Fig. 2C). Since IFN-γ can be produced by both CD8+ and CD4+ T lymphocytes, we measured intracellular levels of IFN-γ in both populations of T cells. We found that both CD4+ and CD8+ T cells with HIF-1α deficiency produce more IFN-γ than wild-type control (Fig. 2D). This confirms that removal of both isoforms of HIF-1α leads to de-inhibition of T cell functions.

**Short isoform I.1 is a minor HIF-1α isoform**

We confirmed and extended our previous observation (20) showing that activation of splenocytes with anti-CD3/anti-CD28 Abs induced expression of I.1 mRNA and, to a lesser
degree, I.2 mRNA (Fig. 3A). To determine proportions of short and long HIF-1α isoforms in purified T lymphocytes, we first activated splenocytes with plate-bound anti-CD3/anti-CD28 mAb, and then separated T cells from B cells and macrophages using auto-MACS magnetic sorter. Surprisingly, even up regulated I.1 mRNA amounts in T cells were approximately 3 times lower than amounts of I.2 mRNA isoform of HIF-1α (Fig. 3B). The observation that I.1 is a minor isoform of HIF-1α was confirmed by Western blot analysis, which showed no significant differences in HIF-1α protein quantity between wild-type and Lck-Cre+/− T cells after the TCR activation (Fig. 3C). These data indicate that despite the enhanced expression in activated T cells, I.1 isoform of HIF-1α represent only a small proportion of total HIF-1α.

Lack of I.1 isoform of HIF-1α results in enhanced secretion of proinflammatory cytokines

Surprisingly, in view of low abundance expression of short I.1 HIF-1α among total HIF-1α molecular species, we found that the lymph node-derived I.1-deficient cells produce more IFN-γ, TNF-α, IL-2, IL-4, and IL-13 (data not shown) upon TCR activation, in both normoxic and hypoxic conditions. The similar effect was observed in splenocytes that were TCR activated by anti-CD3 and anti-CD28 Abs (Fig. 4B). In
addition, I.1 isoform deficiency led to up-regulation of proinflammatory secretion by splenocytes activated by allogenic ML.C. When splenocytes were activated with H-2k4 MHC class I expressing splenocytes from DBA/2 mice (Fig. 4C).

Thus, despite that I.1 isoform represent only a small proportion of HIF-1α, the absence of this minor isoform results in significant up-regulation in production of proinflammatory cytokines.

Conclusion

Our suggestion of the anti-inflammatory role of HIF-1α in T cells was based on our observations of autoimmunity and inflammatory tissue damage in chimeric mice with complete HIF-1α deletion in lymphocytes (8). Here we demonstrate that HIF-1α and activation-inducible short isoform I.1 play an anti-inflammatory role in T cells. Our data suggest that I.1 isoform is disproportionately important in negative regulation of activated T cells and may function as a physiological regulator of T cells by attenuating the T cell activation in a delayed feed-back manner. We suggest that one of the appealing but yet to be tested hypothesis is that increased transcriptional activity of up-regulated HIF-1α and particularly its short isoform may result in higher rates of glycolysis, intracellular acidification, and sub-optimal activities of pH dependent enzymes involved in transmembrane signaling and T cell effector functions. Another possibility is that the short isoform of HIF-1α may inhibit NF-κB activity (e.g., by binding and retaining NF-κB from nuclear translocation, or by up-regulation of expression of IκB), and thereby inhibit proinflammatory transcription. We suggest that HIF-1-mediated anti-inflammatory pathway in T cells is complementary to tissue-protecting immunosuppressive signaling by extracellular adenosine (2, 14, 15, 28, 29), which is accumulatized in hypoxic conditions (30, 31).

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Disclosures

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