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Cutting Edge: Hypoxia-Inducible Factor 1 α 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 1 α (HIF-1 α) 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-1 α ; 2) T cell-targeted HIF-1 α knockdown; and 3) chimeric mice with HIF-1 α gene deletion in T and B lymphocytes. In all three types of mice, the HIF-1 α -deficient T lymphocytes, which were TCR-activated in vitro, produced more proinflammatory cytokines compared with HIF-1 α -expressing control T cells. Surprisingly, deletion of the I.1 isoform, which represents <30% of total HIF-1 α mRNA in activated T cells, was sufficient to markedly enhance TCR-triggered cytokine secretion. These data suggest that HIF-1 α 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)² (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-1 α 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-1 α 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-1 α 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-1 α deletion in cells of adaptive immune system (8). These observations suggested that HIF-1 α 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-1 α in T cells (2) was recently supported by another group showing that HIF-1 α overexpression leads to a decrease in TCR-triggered Ca²⁺ response (11). In addition, HIF-1 α 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-1 α 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-1 α 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-1 α isoform I.2, it retains the DNA-binding and transcriptional activity (20, 23).

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

Materials and Methods

Generation of I.1-deficient mice

HIF-1 α -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

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² Abbreviations used in this paper: HIF-1 α , hypoxia-inducible factor 1 α ; ES cells, embryonic stem cell.

offspring, which were mated with C57BL/6 mice to generate F1 mice. An 0.5-kbp *Bam*HI-*Hind*III fragment containing I.1 exon was replaced with "loxP-I.1 exon-loxP-neo-loxP" cassette, which was subsequently excised by Cre. The targeting vector contained ~1.5-kbp *Xho*I-*Bam*HI 5' homology fragment, 1.5-kbp loxP-I.1 exon-loxP-neo-loxP cassette, 3-kbp *Hind*III-*Eco*RI 3' homology fragment, and diphtheria toxin A chain. DNA was digested with *Sac*I/*Bam*HI and analyzed by Southern blot using ³²P-labeled *Eco*RI-*Sac*I 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-1 α

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

Reagents

All Abs were obtained from BD Biosciences except anti-HIF-1 α 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 μ g/ml streptomycin, 1 mM HEPES, and 50 μ M 2-ME.

RNA preparation and real-time PCR

T lymphocytes were separated from splenocytes, which were incubated with plate-bound anti-CD3 ϵ /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 μ g of total RNA using SuperScript first-strand synthesis kit (Invitrogen Life Technologies) with random hexaprimers. Real-time PCR was performed using SYBR Green master mix (Applied Biosystems) with L32 mRNA as an internal standard. Primers used: I.1 CCCCCTCCACCCATTCT, CTGGCTTCTTGCTTACAGGAGAG; I.2 CACCGATTCCGCATGGA, TTCGACGTCAGAACTCATCTTTT; L32 AGCAACAAGAAACCAAGCAGCAT, TTGACATTGTGGACCAGGAAC.

Results and Discussion

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

We tested the hypothesis that HIF-1 α 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-1 α by developing the HIF-1 α 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 transient transfection of vector expressing Cre recombinase (Fig. 1C). Chimeric mice were bred to produce HIF-1 α -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 to I.1^{+/+} mice (Fig. 1D). For all experiments, I.1^{-/-} and I.1^{+/-} siblings were used, which were produced by breeding of HIF-1 α -I.1^{+/-} heterozygous mice and screened by PCR (Fig. 1E).

Unlike the total knockout of HIF-1 α (27), the disruption of I.1 isoform of HIF-1 α 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

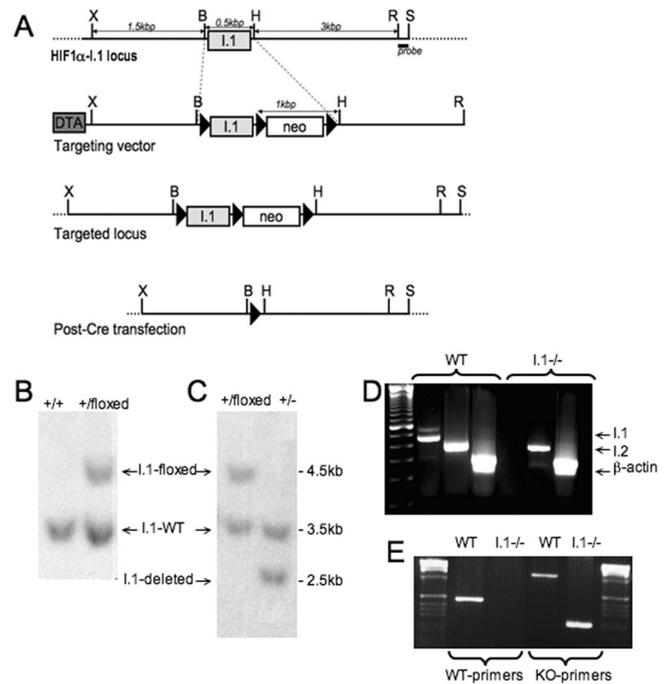


FIGURE 1. Generation of HIF-1 α -I.1-deficient mice. *A*, Strategy used to disrupt I.1 exon of HIF-1 α . *B*, *Bam*HI; *H*, *Hind*III; *R*, *Eco*RI; *S*, *Sac*I; *X*, *Xho*I. *B*, Southern blot analysis of genomic DNA digested with *Bam*HI/*Sac*I from ES after electroporation with targeting vector; *C*, after Cre-mediated deletion of the loxP-flanked I.1 exon and Neo. *D*, RT-PCR analysis of two mRNA isoforms of HIF-1 α from wild-type I.1^{-/-} splenocytes activated with 1 μ g/ml plate-bound anti-CD3 and anti-CD28 mAb for 24 h compared with β -actin mRNA (20). *E*, PCR analysis of genomic DNA obtained from HIF-1 α -I.1^{+/+} and HIF-1 α -I.1^{-/-} mice tails.

differences from wild-type mice as checked by FACS analysis (data not shown).

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

First, we analyzed cytokine production by T cells that lack both isoforms of HIF-1 α . We compared cytokine secretion by TCR-triggered HIF-1 α -deficient CD8⁺ T cells (T cell line HID2) obtained from HIF-1 α -deficient chimeras (8) and the matched HIF-1 α -expressing control CD8⁺ T cells (line 129D2). We found that at both hypoxic (2% O₂) and normoxic (20% O₂) conditions, HIF-1 α -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-1 α -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-1 α -floxed mice, which have T cell-specific deletion of HIF-1 α (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-1 α deficiency produce more IFN- γ than wild-type control (Fig. 2D). This confirms that removal of both isoforms of HIF-1 α leads to de-inhibition of T cell functions.

Short isoform I.1 is a minor HIF-1 α 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

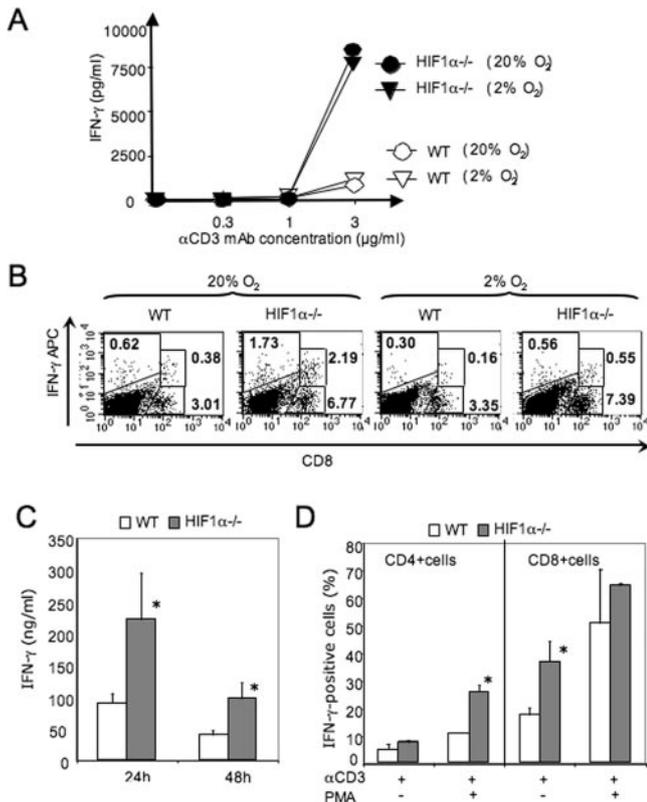


FIGURE 2. Up-regulation of TCR-triggered IFN- γ production in HIF-1 α -deficient T cells. *A*, IFN- γ secretion by HIF-1 $\alpha^{-/-}$ CD8⁺ T cell line HID2 derived from HIF-1 α /RAG-2 chimera spleen cells and normal CTL line 129D2 derived from spleen cells of a 129 mouse, incubated with different concentrations of plate-bound anti-CD3 mAb for 48 h under normoxic conditions (20% O₂) or hypoxic (2% O₂). *B*, Flow cytometry analysis of intracellular IFN- γ production by splenocytes from HIF-1 $\alpha^{-/-}$ chimeras vs 129 mice incubated with 5 μ g/ml plate-bound anti-CD3 mAb for 24 h before addition of 2 μ M monensin. *C*, IFN- γ secretion by Lck-Cre⁺/HIF-1 $\alpha^{-/-}$ T cells compared with wild-type (HIF-1 α -floxed) activated with 0.1 μ g/ml soluble anti-CD3 mAb. *D*, Intracellular IFN- γ production in Lck-Cre⁺/HIF-1 $\alpha^{-/-}$ CD4⁺ and CD8⁺ T cells compared with wild-type cells incubated with 0.1 μ g/ml soluble anti-CD3 for 24 h, followed by addition of 20 ng/ml PMA and 200 ng/ml A23187 for 2 h (where indicated). Quantity of IFN- γ -positive CD4⁺ and CD8⁺ T cells were measured by flow cytometry after preincubation with 10 μ g/ml brefeldin A for 4 h. *, $p < 0.05$ compared with the wild-type.

degree, I.2 mRNA (Fig. 3*A*). To determine proportions of short and long HIF-1 α 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-1 α (Fig. 3*B*). The observation that I.1 is a minor isoform of HIF-1 α was confirmed by Western blot analysis, which showed no significant differences in HIF-1 α protein quantity between wild-type and I.1^{-/-} splenocytes after the TCR activation (Fig. 3*C*). These data indicate that despite the enhanced expression in activated T cells, I.1 isoform of HIF-1 α represent only a small proportion of total HIF-1 α .

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

Surprisingly, in view of low abundance expression of short I.1 HIF-1 α among total HIF-1 α molecular species, we found that

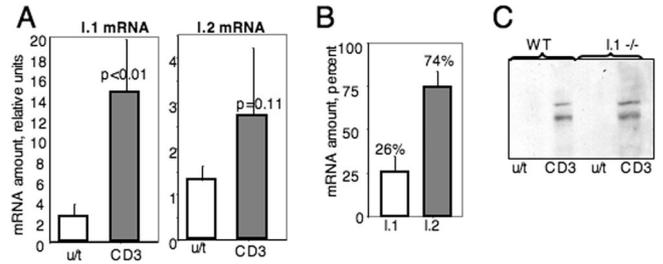


FIGURE 3. I.1 is a minor HIF-1 α isoform. *A*, Real-time RT-PCR analysis of I.1 and I.2 isoforms in activated splenocytes from C57BL/6 mice ($n = 4$) incubated with 1 μ g/ml plate-bound anti-CD3/anti-CD28 mAb for 24 h. *B*, Relative amounts of I.1 and I.2 mRNA isoforms in activated T cells purified from splenocytes of C57BL/6 mice ($n = 3$) measured by real-time RT-PCR. Standard amplification curves for I.1 and I.2 isoforms were calculated using 5 ng of I.1 and I.2 cDNA. *C*, Western blot analysis of HIF-1 α expression in wild-type and I.1^{-/-} splenocytes incubated with or without 1 μ g/ml plate-bound anti-CD3/anti-CD28 mAb for 24 h (ut, untreated; CD3, CD3/CD28-activated).

the lymph node-derived I.1-deficient cells produce more IFN- γ , TNF- α (Fig. 4*A*), 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. 4*B*). In

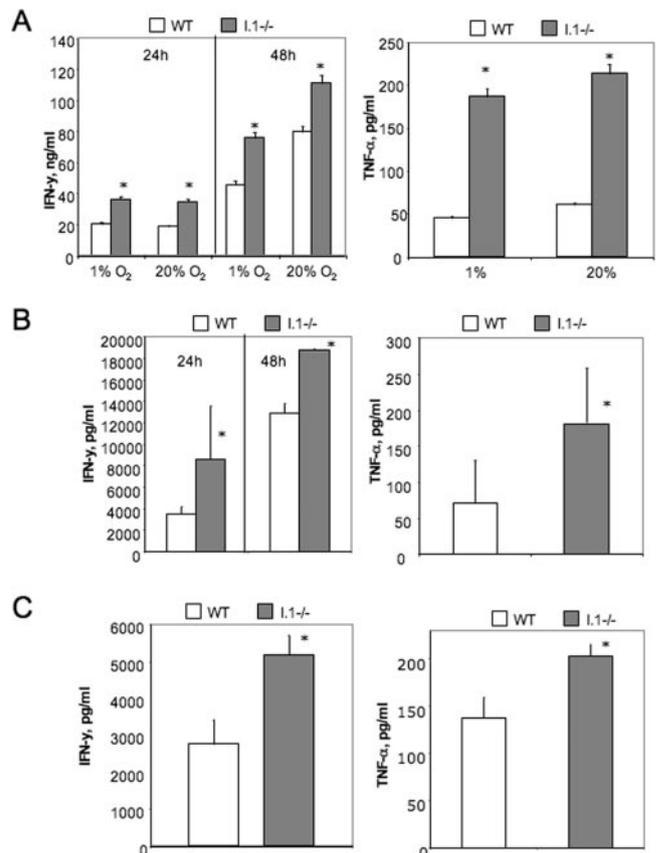


FIGURE 4. Enhanced secretion of proinflammatory cytokines by I.1-deficient immune cells. IFN- γ and TNF- α secretion by immune cells from wild-type ($n = 3$) and I.1^{-/-} mice ($n = 3$) lymph nodes (*A*) and spleens (*B*) activated in triplicates with 1 μ g/ml plate-bound anti-CD3/anti-CD28 mAb for 24 and 48 h, or by mitomycin C-treated DBA/2 splenocytes for 72 h (*C*). *, $p < 0.05$ compared with the wild type. Experiments were repeated at least three times.

addition, I.1 isoform deficiency led to up-regulation of proinflammatory secretion by splenocytes activated by allogenic MLC, when splenocytes were activated with H-2k^d MHC class I expressing splenocytes from DBA/2 mice (Fig. 4C).

Thus, despite that I.1 isoform represent only a small proportion of HIF-1 α , 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-1 α in T cells was based on our observations of autoimmunity and inflammatory tissue damage in chimeric mice with complete HIF-1 α deletion in lymphocytes (8). Here we demonstrate that HIF-1 α 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-1 α and particularly its short isoform may result in higher rates of glycolysis, intracellular acidification, and suboptimal activities of pH dependent enzymes involved in transmembrane signaling and T cell effector functions. Another possibility is that the short isoform of HIF-1 α may inhibit NF- κ B activity (e.g., by binding and retaining NF- κ B from nuclear translocation, or by up-regulation of expression of I κ B), 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 accumulated in hypoxic conditions (30, 31).

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Disclosures

The authors have no financial conflict of interest.

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