Enhanced Interferon- γ Gene Expression in T Cells and Reduced Ovalbumin-Dependent Lung Eosinophilia in Hypoxia-Inducible Factor-1- α -Deficient Mice

Jia Guo^a Wenju Lu^b Larissa A. Shimoda^b Gregg L. Semenza^c
Steve N. Georas^a

^aDivision of Pulmonary and Critical Care, University of Rochester Medical Center, Rochester, N.Y.,

^bDivisions of Pulmonary and Critical Care, Johns Hopkins University, ^cVascular Program, Institute for Cell Engineering, Johns Hopkins University School of Medicine, Baltimore, Md., USA

Key Words

Gene regulation • Transcription Factors • Hypoxia • Asthma mouse model

Abstract

Background: There is growing evidence that hypoxia-inducible transcription factors are involved in the pathophysiology of asthma. Hypoxia-inducible factor-1 α (HIF-1 α) in particular controls the expression of many hypoxia regulated genes, but whether HIF-1 α directly contributes to allergen-driven immune responses is not known. **Methods:** Partially HIF-1 α -deficient mice (HIF-1 $\alpha^{+/-}$) or wild-type littermate controls were used in all experiments. Spleen CD4⁺ T cells were stimulated with anti-CD3 plus anti-CD28 antibodies and cytokine secretion was measured in vitro. Mice were sensitized by intraperitoneal injection of ovalbumin (Ova) plus alum, and then challenged by intranasal Ova followed by bronchoalveolar lavage (BAL) and isolation of spleen cells. BAL cells were counted and the differential determined using cyto-spin, and splenocytes were incubated with Ova to measure recall cytokine production. **Results:** Interferon- γ secretion was significantly higher in anti-CD3 plus anti-CD28 stimulated CD4⁺ T cells obtained from HIF-1 $\alpha^{+/-}$ mice compared to wild-type controls. HIF-1 $\alpha^{+/-}$ mice were protected from lung eosinophilia 72 h after allergen challenge, in as-

sociation with enhanced secretion of interferon- γ in recall responses of splenocytes. **Conclusions:** HIF-1 α contributes to allergic immune responses and lung eosinophilia in a mouse model of asthma.

Copyright © 2009 S. Karger AG, Basel

Background

Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcription factor composed of HIF-1 α and HIF-1 β subunits that each contain basic helix-loop-helix and PAS domains, which mediate heterodimerization and DNA binding [1]. Under hypoxic conditions, the degradation of HIF-1 α is inhibited, resulting in nuclear accumulation of the protein, dimerization with constitutively expressed HIF-1 β , and binding to hypoxia response elements (HREs) leading to transcriptional activation of target genes via recruitment of coactivators such as p300 and CBP [1, 2]. Hypoxia independent signals that activate HIF-1 α under normoxic conditions are also being uncovered, including transforming growth factor- β and other cytokines and growth factors [3, 4].

There is growing evidence that hypoxia-inducible transcription factors contribute to the pathophysiology of asthma. For example, a proteomic analysis identified

upregulation of several HIF-1 dependent target genes in a mouse model of asthma, including the glycolytic enzymes aldolase A, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase and enolase 1 [5]. Other potential HIF-1 α targets in asthma include vascular endothelial growth factor (VEGF), which is increased in the airway in asthmatics and causes angiogenesis [6], and MUC5AC [7]. The expression of HIF-1 α is increased in airway biopsies from asthmatics [8] and also in activated mast cells [9]. Furthermore, a novel thiol compound was recently shown to attenuate allergic lung inflammation in mice, in association with reduced HIF-1 α expression [10]. Despite these indirect associations, there is currently little direct evidence that HIF-1 α contributes to airway inflammation or allergic immune responses.

Complete deficiency of HIF-1 α in mice results in embryonic lethality at midgestation that is associated with cardiovascular and neural tube defects [11], as well as abnormalities in B cell development after blastocyst complementation [12]. Mice that are heterozygous for a *Hif1a* knockout allele develop normally but have impaired physiological responses to hypoxia and ischemia [13–15]. Here we investigated T cell cytokine gene expression using heterozygous HIF-1 α ^{+/-} mice in a mouse model of asthma.

Methods

Animal Care

Female HIF-1 α ^{+/-} mice, and wild-type littermate controls on the C57BL/6 $\times$ 129 background between 6–8 weeks of age were used in all experiments. Experiments were conducted blinded to genotype at the Johns Hopkins University School of Medicine in compliance with the animal care and use committee guidelines.

Flow Cytometry and Western Blot

We first analyzed the cellularity and composition of the thymus, peripheral lymph nodes, and spleen in wild-type and HIF-1 α ^{+/-} littermates using immunofluorescence and flow cytometry with antibodies directed against CD3, CD4, CD8, B220 and NK1.1 (all from Pharmingen) and a CellQuest flow cytometer. HIF-1 α protein was analyzed in whole cell splenocyte lysates (40 μ g/lane) using a commercially available antibody (Abcam) as described [2].

Cell Isolation and Stimulation

Spleen CD4⁺ T cells were isolated using negative immunomagnetic selection and commercially available kits according to the manufacturer's instructions (Miltenyi, Biotec). Cells were cultured in RPMI-1640 (1 $\times$ 10⁶/ml) supplemented with 10% fetal calf serum (Hyclone) and stimulated with plate-bound anti-CD3 antibodies (5 μ g/ml, Invitrogen) plus soluble anti-CD28 (5 μ g/ml, Invitrogen) for 72 h. Cell-free supernatants were analyzed by

ELISA using commercially available kits [interleukin (IL)-4, 5, 6 and interferon (IFN)- γ ; R&D Systems].

Mouse Asthma Model

Female mice were sensitized with endotoxin-free ovalbumin (Ova; Grade V, Sigma, 20 μ g) adsorbed to aluminum hydroxide (Pierce) by intraperitoneal (i.p.) injection on days 0 and 14, followed by intranasal (i.n.) challenge with ovalbumin solution (2.5 μ g in 75 μ l phosphate buffered saline, PBS) on days 28, 29 and 30. On day 33 (72 h after the last challenge), mice were sacrificed, bronchoalveolar lavage (BAL) was performed with 1 ml aliquots of PBS, and splenocytes were isolated using sterile techniques. BAL fluids were analyzed by cyto-spin and Diff-Quick staining, and spleen cells were incubated with ovalbumin (5 μ g) for 72 h. Lung tissues were analyzed using routine histology and hematoxylin and eosin staining. An aliquot of total splenocyte RNA was analyzed by reverse transcription PCR using primers specific for the lineage-specific transcription factor GATA3 and GAPDH as control. Cell-free supernatants were analyzed for cytokine secretion by ELISA using commercially available kits, as above.

Results

There were no differences in the numbers or composition of CD4⁺, CD8⁺, or CD4⁺CD8⁺ lymphocytes in the thymus of HIF-1 α ^{+/-} mice compared to wild-type controls, and similarly no differences in the number or composition of spleen or peripheral lymph node T or B cells (data not shown). We confirmed that HIF-1 α protein expression was reduced in HIF-1 α ^{+/-} splenocytes (fig. 1a), and concluded that partial deficiency of HIF-1 α does not grossly perturb lymphocyte development.

Lukashev et al. [16] and colleagues recently reported that a HIF-1 isoform inhibited T cell activation. Therefore, we next investigated whether partial HIF-1 α ^{+/-} deficiency affected T cell activation in vitro. To test this possibility, we isolated CD4⁺ T cells from wild-type or HIF-1 α ^{+/-} mice and stimulated them with antibodies directed against the T cell receptor and CD28. Figure 1b shows that after 72 h, CD4⁺ T cells from HIF-1 α ^{+/-} mice released significantly more IFN- γ than their wild-type counterparts ($p < 0.05$), whereas IL-4 production was not significantly different between genotypes (fig. 1c, $p =$ not significant).

We next wanted to determine the effects of partial HIF-1 α deficiency on an allergen-driven immune response. To investigate this, we used wild-type and HIF-1 α ^{+/-} littermates in a standard mouse model of asthma using ovalbumin as the model allergen (see Methods). After Ova sensitization and challenge, eosinophils were readily detectable in BAL from wild-type mice and sig-

Fig. 1. Higher IFN- γ production in HIF-1 $\alpha^{+/-}$ T cells. **a** Whole cell extracts from wild-type (WT) or HIF-1 $\alpha^{+/-}$ (HET) spleen CD4+ T cells were analyzed by Western blot for HIF-1 α and β -actin protein expression (n = 2 each). **b, c** Spleen CD4+ T cells from

naïve mice were stimulated with antibodies directed against CD3 and CD28 for 72 h. Data are the mean $\pm$ SEM of cytokine production as determined by ELISA (sensitivity <7.5 pg/ml) using n = 6 mice per genotype. * p < 0.05.

Fig. 2. HIF-1 $\alpha^{+/-}$ (HET) mice are protected from BAL eosinophilia after sensitization and challenge with ovalbumin. **a** The percentages of eosinophils (Eos), neutrophils (Neuts), macrophages (Macs), and lymphocytes (Lymph) recovered 48 h after last challenge are shown. Control mice were sensitized and challenged with saline alone. Results are the mean $\pm$ SEM of n = 10 mice per genotype. * p < 0.05, saline vs. ovalbumin; # p < 0.05, wild-type (WT) vs. HET. **b, c** Splenocytes from Ova-sensitized and challenged WT and HET were incubated in vitro with Ova for 72 h, and IL-4 (**b**) and IFN- γ (**c**) secretions were measured by ELISA. Results are the mean $\pm$ SEM of n = 10 mice per genotype. * p < 0.05. **d** Splenocyte mRNA was analyzed for expression of GATA3 or GAPDH by RT-PCR after culture with or without Ova, as shown. The figure is from 1 experiment representative of n = 6 mice.

nificantly compared to HIF-1 $\alpha^{+/-}$ mice (fig. 2a). This was not accompanied by differences in the total number of inflammatory cells recruited to the lungs, and although there was a trend towards greater BAL neutrophilia in HIF-1 $\alpha^{+/-}$ mice compared to wild-type controls (29 vs. 22%) this did not reach statistical significance (fig. 2a and data not shown). Similar results were observed using lung

tissues where we observed greater numbers of peri-bronchial and perivascular eosinophils in wild-type versus HIF-1 $\alpha^{+/-}$ littermates (data not shown).

In order to determine if reduced BAL eosinophilia was due to a less Th2-biased immune response in HIF-1 $\alpha^{+/-}$ mice, we first analyzed BAL supernatants for IL-4 and IFN- γ . The levels of these cytokines were at or below the

detection limit (7.5 pg/ml) for each cytokine tested, possibly reflecting strain- or protocol-dependent factors. However, in recall challenge assays in which splenocytes from wild-type and HIF-1 α ^{+/-} littermate mice were restimulated with Ova, we found that IFN- γ production, which was below the detection limit using wild-type cells, was significantly enhanced in HIF-1 α ^{+/-} splenocytes, whereas IL-4 production was unaffected by genotype (fig. 2b, c). Similarly, equal amounts of IL-5 were produced by wild-type and HIF-1 α ^{+/-} splenocytes in response to Ova (35 $\pm$ 2.3 vs. 38 $\pm$ 4.6 pg/ml, respectively). Expression of mRNA of the Th2 lineage marker GATA3 did not differ between groups (fig. 2d). These data suggest that reduced BAL eosinophilia in HIF-1 α ^{+/-} mice may be due to excess production of IFN- γ , a cytokine that counteracts allergen-driven eosinophilia.

Discussion

Although there is growing evidence that hypoxia-inducible transcription factors contribute to the pathophysiology of asthma [5, 6], very little is currently known about whether or how HIF-1 α regulates allergic immune responses. We show using gene-targeted mice that partial deficiency of HIF-1 α results in: (1) higher secretion of IFN- γ from both CD4⁺ T cells activated polyclonally as well as splenocytes activated in an antigen-specific fashion, and (2) attenuated BAL eosinophilia in a mouse model of asthma. These novel data suggest that by repressing IFN- γ HIF-1 α might promote allergen-specific immune responses in a T cell intrinsic manner.

In the context of a normal immune response, T cells will be exposed to hypoxic tissue microenvironments as they traverse secondary lymphoid organs such as the spleen, where very low oxygen tensions have been recorded [17]. In addition, HIF-1 α can also be activated under normoxic conditions by cytokines and growth factors [3, 4]. Emerging data suggest that hypoxia and HIF-1 regulate several aspects of T cell biology. For example, hypoxia potentiates T cell survival [17], which was recently attributed to HIF-1-mediated expression of adrenomedullin and inhibition of activation-induced cell death [18]. Hypoxia also downregulates T-cell Kv1.3 channels and can affect Ca²⁺ signaling [19, 20], although it is currently not known whether these are HIF-1-dependent effects.

Our data add to growing literature indicating that HIF-1 α is a negative regulator of T cells. For example, using an elegant T cell specific conditional deletion strategy, Thiel et al. [21] showed that HIF-1 α deficiency result-

ed in enhanced T cell activation in a mouse model of sepsis. Exactly how HIF-1 α regulates T cell activation and IFN- γ secretion remains to be determined. We found that partial HIF-1 α deficiency enhanced allergen-driven IFN- γ secretion without affecting expression of the Th2/Th1 markers GATA3 or T-bet (fig. 2; data not shown). This suggests that HIF-1 α might directly repress cytokine gene secretion per se more than T helper differentiation, and are in keeping with the recent report of Lukashev et al. [16]. We found that HIF-1 α did not regulate activity of a 312 base pair human IFN- γ promoter reporter construct, suggesting that the repressive effects of HIF-1 α are not mediated at the level of promoter activation (data not shown). Therefore, the precise mechanism(s) by which HIF-1 α regulates IFN- γ expression remain to be determined. It will be interesting in future studies to examine the effects of HIF-1 α on T cell lineage commitment, including possible effects on the development or function of regulatory T cells.

We used mice that were partially deficient in HIF-1 α , since complete deficiency of this factor results in embryonic lethality. Previous studies in other models have shown that partial deficiency of HIF-1 α is sufficient to attenuate responses to hypoxia and ischemia [13–15]. For example, Li et al. [14] found that HIF1- α ^{+/-} were protected from metabolic derangements induced by intermittent hypoxia in association with alterations in cytokine production in adipocytes. Taken together with our findings, these data indicate that HIF-1 α regulates diverse cellular responses in a gene-dosage dependent manner. A likely explanation for reduced lung eosinophilia we report is the known inhibitory effect of IFN- γ on eotaxin-3 gene expression [22]. Future studies in which the expression of HIF-1 α can be regulated in T cells should help clarify the role of this factor in allergic immune responses.

In summary, we report novel data implicating the transcription factor HIF-1 α in the allergic immune response to Ova. Partial deficiency of HIF-1 α results in attenuated lung eosinophilia in association with higher allergen-driven IFN- γ production. Our data suggest that HIF-1 α normally serves to repress IFN- γ expression in a T cell intrinsic manner, and that inhibiting HIF-1 α may be beneficial in treating allergic lung inflammation.

Acknowledgements

Funding sources: NIH R01 HL073952 (to S.G.) and NIH R01 HL55338 (to G.S.).

References

- 1 Semenza GL: Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 2003;3:721–732.
- 2 Fukuda R, Zhang H, Kim JW, Shimoda L, Dang CV, Semenza GL: HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells. *Cell* 2007;129:111–122.
- 3 McMahon S, Charbonneau M, Grandmont S, Richard DE, Dubois CM: Transforming growth factor beta1 induces hypoxia-inducible factor-1 stabilization through selective inhibition of PHD2 expression. *J Biol Chem* 2006;281:24171–24181.
- 4 Phillips RJ, Mestas J, Gharaee-Kermani M, Burdick MD, Sica A, Belperio JA, Keane MP, Strieter RM: Epidermal growth factor and hypoxia-induced expression of CXCR4 chemokine receptor 4 on non-small cell lung cancer cells is regulated by the phosphatidylinositol 3-kinase/PTEN/AKT/mammalian target of rapamycin signaling pathway and activation of hypoxia inducible factor-1alpha. *J Biol Chem* 2005;280:22473–22481.
- 5 Fajardo I, Svensson L, Bucht A, Pejler G: Increased levels of hypoxia-sensitive proteins in allergic airway inflammation. *Am J Respir Crit Care Med* 2004;170:477–484.
- 6 Hoshino M, Nakamura Y, Hamid QA: Gene expression of vascular endothelial growth factor and its receptors and angiogenesis in bronchial asthma. *J Allergy Clin Immunol* 2001;107:1034–1038.
- 7 Young HW, Williams OW, Chandra D, Bellinghausen LK, Perez G, Suarez A, Tuvim MJ, Roy MG, Alexander SN, Moghaddam SJ, Adachi R, Blackburn MR, Dickey BF, Evans CM: Central role of Muc5ac expression in mucous metaplasia and its regulation by conserved 5' elements. *Am J Respir Cell Mol Biol* 2007;37:273–290.
- 8 Lee SY, Kwon S, Kim KH, Moon HS, Song JS, Park SH, Kim YK: Expression of vascular endothelial growth factor and hypoxia-inducible factor in the airway of asthmatic patients. *Ann Allergy Asthma Immunol* 2006;97:794–799.
- 9 Walczak-Drzewiecka A, Ratajewski M, Wagner W, Dasty J: HIF-1alpha is up-regulated in activated mast cells by a process that involves calcineurin and NFAT. *J Immunol* 2008;181:1665–1672.
- 10 Lee KS, Kim SR, Park HS, Park SJ, Min KH, Lee KY, Choe YH, Hong SH, Han HJ, Lee YR, Kim JS, Atlas D, Lee YC: A novel thiol compound, N-acetylcysteine amide, attenuates allergic airway disease by regulating activation of NF-kappaB and hypoxia-inducible factor-1alpha. *Exp Mol Med* 2007;39:756–768.
- 11 Kotch LE, Iyer NV, Laughner E, Semenza GL: Defective vascularization of HIF-1alpha-null embryos is not associated with VEGF deficiency but with mesenchymal cell death. *Dev Biol* 1999;209:254–267.
- 12 Kojima H, Gu H, Nomura S, Caldwell CC, Kobata T, Carmeliet P, Semenza GL, Sitkovsky MV: Abnormal B lymphocyte development and autoimmunity in hypoxia-inducible factor 1alpha-deficient chimeric mice. *Proc Natl Acad Sci USA* 2002;99:2170–2174.
- 13 Yu AY, Shimoda LA, Iyer NV, Huso DL, Sun X, McWilliams R, Beaty T, Sham JS, Wiener CM, Sylvester JT, Semenza GL: Impaired physiological responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1alpha. *J Clin Invest* 1999;103:691–696.
- 14 Li J, Bosch-Marce M, Nanayakkara A, Savransky V, Fried SK, Semenza GL, Polotsky VY: Altered metabolic responses to intermittent hypoxia in mice with partial deficiency of hypoxia-inducible factor-1alpha. *Physiol Genomics* 2006;25:450–457.
- 15 Cai Z, Zhong H, Bosch-Marce M, Fox-Talbot K, Wang L, Wei C, Trush MA, Semenza GL: Complete loss of ischaemic preconditioning-induced cardioprotection in mice with partial deficiency of HIF-1 alpha. *Cardiovasc Res* 2008;77:463–470.
- 16 Lukashev D, Klebanov B, Kojima H, Grinberg A, Ohta A, Berenfeld L, Wenger RH, Sitkovsky M: Cutting edge: hypoxia-inducible factor 1 alpha and its activation-inducible short isoform I.1 negatively regulate functions of CD4+ and CD8+ T lymphocytes. *J Immunol* 2006;177:4962–4965.
- 17 Caldwell CC, Kojima H, Lukashev D, Armstrong J, Farber M, Apasov SG, Sitkovsky MV: Differential effects of physiologically relevant hypoxic conditions on T lymphocyte development and effector functions. *J Immunol* 2001;167:6140–6149.
- 18 Makino Y, Nakamura H, Ikeda E, Ohnuma K, Yamauchi K, Yabe Y, Poellinger L, Okada Y, Morimoto C, Tanaka H: Hypoxia-inducible factor regulates survival of antigen receptor-driven T cells. *J Immunol* 2003;171:6534–6540.
- 19 Conforti L, Petrovic M, Mohammad D, Lee S, Ma Q, Barone S, Filipovich AH: Hypoxia regulates expression and activity of Kv1.3 channels in T lymphocytes: a possible role in T cell proliferation. *J Immunol* 2003;170:695–702.
- 20 Robbins JR, Molleran Lee S, Filipovich AH, Szigligeti P, Neumeier L, Petrovic M, Conforti L: Hypoxia modulates early events in T cell receptor-mediated activation in human T lymphocytes via Kv1.3 channels. *J Physiol* 2005;564:131–143.
- 21 Thiel M, Caldwell CC, Kreth S, Kuboki S, Chen P, Smith P, Ohta A, Lentsch AB, Lukashev D, Sitkovsky MV: Targeted deletion of HIF-1alpha gene in T cells prevents their inhibition in hypoxic inflamed tissues and improves septic mice survival. *PLoS ONE* 2007;2:e853.
- 22 Heller NM, Matsukura S, Georas SN, Boothby MR, Rothman PB, Stellato C, Schleimer RP: Interferon-gamma inhibits STAT6 signal transduction and gene expression in human airway epithelial cells. *Am J Respir Cell Mol Biol* 2004;31:573–582.