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HIF-1α influences myeloid cell antigen presentation and response to subcutaneous OVA vaccination

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Abstract Hypoxia-inducible factor (HIF)-1 is a transcription factor known to play an important role in regulating the innate immune response to infection. Under baseline conditions, cellular HIF-1 levels in leukocytes are scarce, but levels rise rapidly in response to hypoxia or molecular signals of infection or inflammation such as microbial surface molecules and host-derived cytokines. Innate immune cells such as macrophages, neutrophils, and mast cells exhibit increased microbicidal activity when HIF-1 levels are increased, and mice lacking HIF-1 are more susceptible to invasive bacterial infection. In this study, we used genetic and pharmacologic means to determine whether HIF-1 also plays an important role in the adaptive immune response to infection. HIF-1α/Tie-2 Cre^{+} mice harboring a >90 % knockdown of HIF-1 in myeloid cells were studied. We found antigen-presenting cells from these mice that expressed lower levels of MHC-II and the costimulatory molecules CD80 and CD86, and were less able to induce T cell proliferation. These differences were present at baseline and persisted after activation. Increasing HIF-1 levels in wild-type (WT) cells by using the prolyl hydroxylase inhibitor drug AKB-4924 had the opposite effect, increasing MHC and costimulatory molecule expression and T cell proliferation. In experimental vaccination, HIF-1α/Tie-2 Cre^{+} mice exhibited a weaker T cell response and lower antibody levels in response to vaccination than WT mice, while WT mice treated with a drug to elevate HIF-1 responded more strongly to vaccination. Thus, HIF-1 participates in bridging the innate and adaptive immune responses and may merit further exploration as an adjuvant target.

Keywords Hypoxia-inducible factor · Dendritic cell · Antigen presentation · Adjuvant · Vaccination

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Introduction

Antibiotics and childhood vaccinations drastically lowered the burden of infectious diseases in the twentieth century, but in recent decades, progress in combating infectious diseases has slowed. A better understanding of the factors which control the immune response to infection would help us design treatments or vaccine adjuvants that would improve our ability to control infectious diseases. Hypoxia-inducible factor (HIF) has been called a "master regulator" of innate immune function [1] because it plays a crucial role in enhancing the bactericidal activity of myeloid cells such as macrophages and neutrophils [2]. Its role in the adaptive immune response to infection has to date received less attention.

HIF is a basic helix–loop–helix transcription factor [3] first identified because of its role in erythropoietin regulation [4]. The transcriptional factor is a heterodimer composed of HIF-α and HIF-β subunits [3]. HIF-α is actually a family of three genes: HIF-1α, HIF-2α, and HIF-3α, of which HIF-1α is the best understood. HIF-3α is distantly related to HIF-1α and HIF-2α [5], and little is known about its function, although it may inhibit the activity of HIF-1α and HIF-2α [6]. The HIF-1α and HIF-2α subunits are closely related [7], although they differ in tissue distribution:

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HIF-1α is ubiquitously expressed, whereas HIF-2α is most abundantly expressed in vascular endothelial cells during embryonic development [7].

Both HIF-α and HIF-β are constitutively produced, but in the presence of oxygen, HIF-α is rapidly degraded by oxygen-dependent mechanisms [8]. In the absence of oxygen, HIF-α is not degraded and instead translocates into the nucleus where it dimerizes with HIF-β and binds to hypoxia-response elements in the promoter regions of more than 70 genes [9]. HIF has been shown to regulate genes involved in glycolysis, angiogenesis, cell differentiation, apoptosis, and others [8, 9].

Sites of infection and inflammation are hypoxic microenvironments, with oxygen tensions reported under 1 % [10]. The low oxygen tension is due to the combination of reduced perfusion due to physical damage and a high density of activated inflammatory cells that place a high metabolic load on the area. Consequently, innate immune cells must execute their key antimicrobial functions under hypoxic conditions. HIF1-deficient myeloid cells exhibit reduced levels of cellular ATP (15–40 % of normal), emphasizing the essential role of the transcriptional regulator for energy production through glycolysis in these immune cells [2]. Through HIF-1 induction under conditions of hypoxia, myeloid cells release more nitric oxide, granule proteases, and antimicrobial peptides; survive longer because of reduced apoptosis; and kill Gram-positive and Gram-negative bacterial pathogens more efficiently than at normoxia [11, 12]. HIF activation is apparent during the differentiation of circulating monocytes into tissue macrophages [13] and may promote phagocytic uptake of bacteria under hypoxia [14].

Myeloid cells such as macrophages and dendritic cells (DC) play a crucial role in triggering the adaptive response to infection through their function as antigen-presenting cells (APCs), but our understanding of the role of HIF-1 in this critical aspect of host defense is much less developed. Research to examine potential roles of HIF in APCs has yielded contradictory results (reviewed by [15]), and much of the previous work has focused more closely upon the effects of hypoxia per se, rather than HIF transcriptional regulation, on DC activities. While some investigators have produced data that would indicate that hypoxia inhibits DC differentiation, maturation markers, and antigen capture [16–18], others have come to exactly the opposite conclusion, namely that hypoxia promoted DC maturation both alone [19] and in combination with lipopolysaccharide (LPS) stimulus [17].

Hypoxia and HIF induction cannot be approached as functional equivalents in studies of the immune responses, because HIF is also induced at the transcriptional level by a variety of signals other than hypoxia, including markers of infection and inflammation such as cytokines [20, 21] and viral proteins [22, 23]. Furthermore, when HIF is activated by hypoxia, its contribution to transcriptional regulation comprises a different set of target genes than when it is activated by a TLR ligand such as LPS [24].

Here, we contribute a new approach to our understanding of HIF in DC biology, by employing the genetic system of myeloid HIF-1α-null-deficient mice. Coupled with pharmacological studies with a potent HIF-1-specific pharmacological agonist, we examine antigen presentation function in vitro and in vivo, lending support to a significant role of HIF regulation in these phenotypes.

Methods

Cell culture

Bone marrow (BM) was obtained from the hind legs of mice age 8–16 weeks and grown in RPMI with 10 % endotoxin-free FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamicin, 50 μM β_2-mercaptoethanol, 10 % conditioned media from GM-CSF-expressing B16 cells, and 20 ng/ml IL-4 (Peprotech). Media was changed on day 3 and 5 and the nonadherent cells were used between days 8–11. RAW264.7 macrophages were grown in Roswell Park Memorial Institute medium (RPMI) with 10 % FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin.

Surface marker analysis

BM-derived DC from HIF-1α/Tie-2 Cre^+ or Cre^- mice differentiated for 8–11 days were plated in triplicate at 10^6 cells/well in 12-well plates and treated with LPS (10 or 100 ng/ml, as indicated), TNF (500 U/ml), or media alone, overnight. RAW264.7 macrophages were also plated in triplicate at 10^6 cells/well in 12-well plates and treated with AKB-4924 (10 or 100 μM, as indicated), the vehicle for AKB-4924 (cyclodextrin, at equivalent concentration), both LPS (100 ng/ml) and 4924, or vehicle alone overnight. The following day, the nonadherent DC or RAW macrophages were harvested, washed, and stained with the fluorescent anti-mouse antibodies CD11c-FITC, MHC-II-PE, CD86-PECy7, and CD80-APC. Fluorescence was measured by flow cytometry (FACSCalibur, BD Biosciences) and analyzed using FlowJo (TreeStar). Mean fluorescence was calculated on cells gated on $CD11c^+$ cells.

Cytokine protein measurement

BM-derived DC from HIF-1α/Tie-2 Cre^+ or Cre^- mice differentiated for 8–11 days were plated in triplicate at 10^6 cells/well in 12-well plates and treated with LPS (10 or 100 ng/ml, as indicated) or media alone, overnight. For

some experiments, BM-derived DC from C57BL/6 mice (Charles River) between days 8–11 of differentiation were treated with AKB-4924 (10 or 100 μM, as indicated), the vehicle for AKB-4924 (cyclodextrin, at equivalent concentration), both LPS (10 ng/ml) and AKB-4924, or vehicle alone overnight. The following day, the supernatant was harvested and protein levels measured by ELISA (IL-6, BD Biosciences no. 555240; IL-10, BD Biosciences no. 555252; IL-12 p70, BD Biosciences no. 555256; TNF-α, R&D Biosciences no. DY410).

Cytokine RT-PCR

BM-derived DC from HIF-1α/Tie-2 Cre^+ or Cre^- mice that had been differentiated for 8 to11 days were plated in triplicate at 10^6 cells/well in 12-well plates and treated with LPS (10 or 100 ng/ml, as indicated) or media alone for 4 h. For some experiments, bone marrow-derived dendritic cells from C57BL/6 mice (Charles River) between day 8 and day 11 of differentiation were treated with AKB-4924 (10 or 100 μM, as indicated), the vehicle for AKB-4924 (cyclodextrin, at equivalent concentration), both LPS (10 ng/ml) and 4924, or vehicle alone for 4 h. RNA was obtained using the RNEasy Mini kit (Qiagen, cat no. 74104), reverse transcribed to DNA using the iScript cDNA synthesis kit (BioRad, cat no. 170-8890), and RT-PCR run using iQ SYBR Green Supermix (BioRad, cat no. 170-8882). RT-PCR conditions: 50 °C for 10 min, 95 °C for 5 min, 40 cycles of 95 °C for 10 s followed by 56 °C for 30 s, 95 °C for 10 s, and a melt curve from 55 °C to 90 °C in increments of 0.5 °C for 5 s each. Data were normalized using β-actin. Primer sequences and annealing temperatures used for RT-PCR analysis of β-actin (housekeeping control), IL-6, IL-8, IL-10, IL-12, and TNF-α are available on request.

T cell proliferation assay

BM-derived DC from HIF-1α/Tie-2 Cre^+ or Cre^- mice differentiated for 8–11 days were harvested, washed, plated at 2×10^4 cells/well in a 96-well plate, and incubated overnight with the OT-I peptide SIINFEKL at 20 or 0 μg/ml, or with the OT-II peptide $OVA_{323-337}$ at 20 or 0 μg/ml. The next day, CD8 T cells were obtained from the spleen and lymph nodes of naïve OT-I mice, and $CD4^+$ T cells were obtained from the spleen and lymph nodes of naïve OT-II mice using MACS purification kits (Miltenyi Biotec, $CD4^+$ cat no. 130-095-248; CD8α+ cat no. 130-095-236). The T cells were added at 2×10^5 cells/well (a 1:10 DC/T cell ratio) in RPMI with 10 % FBS, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μM β-mercaptoethanol, 10 mM HEPES, and 100 μM MEM-NEAA. After 3 days of incubation, 0.25 μCi/well of ^{3}H-thymidine was added, and the plate was incubated for an additional 8–16 h, at which point the cells were lysed by freezing at −80 °C and read on an automated harvester (Becton Dickinson).

Vaccination experiments

HIF-1α/Tie-2 Cre^+ mice and age- and sex-matched Cre^- controls were immunized subcutaneously on the back with 50 μg antigen (SIINFEKL or OVA) in 50 μl PBS emulsified with 50 μl IFA, for a total volume of 100 μl. In other experiments, wild-type C57BL/6 mice were treated with 5 mg/kg AKB-4924 or vehicle control by intraperitoneal injection 24 h before, 1 h before, and 16 h after immunization as above. The SIINFEKL-immunized mice were sacrificed on day 8; spleens were harvested, plated at 2×10^6 cells/well in a 96-well ELISPOT plate precoated with anti-IFNγ antibody, then restimulated with SIINFEKL at 2.5 μg/ml or with irrelevant OT-II peptide as a negative control. The number of IFNγ-producing cells in the whole splenocyte population was measured by ELISPOT (Mabtech). The OVA-immunized mice were bled on day 14 after immunization. ELISA plates were coated with 2 μg/ml OVA in PBS, and the OVA-specific IgG serum antibody titer was measured using anti-mouse IgG HRP-linked antibodies.

Statistical analysis

Statistical significance was calculated by *t* test or two-way ANOVA, as appropriate for the data, using GraphPad Prism.

Results

DC provide three signals to T cells: the antigen, presented in the context of MHC-I or MHC-II; costimulatory signals, achieved through ligation of surface molecules; and lastly, the release of cytokines and other soluble mediators. The combination of signals alerts the T cells to the foreign antigen, activates them, and modulates the strength and polarization of the adaptive immune response. To find out what role HIF plays in the ability of APCs to stimulate the adaptive immune response, we used HIF-1α/Tie-2 Cre mice. These mice harbor a targeted deletion of HIF-1α in endothelial cells and hematopoietic precursors; HIF-1α/Tie2-Cre line exhibits 98 % deletion efficiency in bone marrow cells [25].

We first looked at the effect of HIF-1α ablation on the DC lineage itself. BM-derived DC from HIF-1α KO mice and Cre- controls showed decreased expression of key surface molecules. Immature DC lacking HIF expressed less MHC-II and the costimulatory molecules CD80 and CD86 at baseline, and when they were activated by overnight

exposure to LPS, the deficit persisted (Fig. 1a). Likewise, DC that had been matured by exposure to TNF-α also expressed lower levels of MHC-II, CD80, and CD86 (Fig. 1b). These findings suggest that HIF-1α is important for the ability of activated DC to deliver the first and second activation signals to T cells.

In contrast, we did not identify a requirement for HIF-1α in the LPS-stimulated release of cytokines by DC. Key cytokines known to play a role in the adaptive immune response were examined. IL-6 is a B cell differentiation factor, and it also may play a role in polarizing the T helper cell response, while IL-12 is a proinflammatory cytokine that promotes Th1 polarization and IL-10 is an anti-inflammatory cytokine that promotes Th2 polarization [26, 27]. TNF-α enhances the proliferation of T cells. No differences were found for any of these cytokines at the level of protein (Fig. 2a) or mRNA transcript (Fig. 2b). This result distinguishes DC from other myeloid cells such as macrophages and neutrophils, in which we reported that deletion of HIF-1α modulated the release of certain proinflammatory cytokines [11].

As a primary function of DC is to activate T cells, we next asked whether DC lacking HIF-1α were able to stimulate T cell proliferation. BM-derived DC were coated with the MHC-I-restricted peptide SIINFEKL or the MHC-II-restricted peptide $OVA_{323–337}$, and then incubated with $CD8^+$ T cells or $CD4^+$ T cells from OT-I or OT-II mice, respectively. Both $CD4^+$ and $CD8^+$ T cells incubated with KO DC exhibited significantly less proliferation than those incubated with Cre^- control DC (Fig. 3a, b), indicating that HIF-1α plays an important role in the ability of DC to trigger T cell proliferation.

Since the loss of HIF-1α impairs the ability of APCs to present antigen, provide costimulatory signals, and stimulate T cell proliferation in vitro, we hypothesized that it should also impair the in vivo response to vaccination. Indeed, when HIF-1α KO mice were vaccinated with the ovalbumin subpeptide SIINFEKL, they produced fewer antigen-specific T cells than Cre^- control mice (Fig. 3c). However, when serum antibody titers were examined, the effect was weaker. Although a trend of diminished response could be discerned, the effect of HIF-1α ablation on serum IgG titer in response to vaccination with OVA did not reach statistical significance (Fig. 3d).

Our evidence in the genetic model systems to this point suggested that loss of HIF-1α impairs the function of DC in promoting adaptive immune response. For the purpose of preventing or treating infectious disease, one could contemplate whether pharmacological agents that increase cellular HIF-1α levels could improve DC function in the immune response. Proof-of-principle studies were performed with RAW264.7 macrophages, showing that when HIF levels are increased with the HIF-1α-boosting drug AKB-4924 [28], the macrophages express higher levels of MHC-II, CD80, and CD86 (Fig. 4a), a result in agreement with the genetic studies in Fig. 1. AKB-4924 inhibits the prolyl hydroxylases [28] that negatively regulate HIF-1α [29] and preferentially elevates HIF-1α over HIF-1β [28]. Also, in agreement with our data from the knockout mice, we found that AKB-4924 had no effect on cytokine release from WT BM-derived DC (Fig. 4b), but did strengthen both the cell-mediated (Fig. 4c) and humoral (Fig. 4d) response to OVA immunization.

Discussion

The data presented above indicate that HIF-1α plays a modest but discernible role in the ability of DC to trigger the adaptive immune response to infection. Loss of HIF-1α reduces the surface expression of major histocompatibility and costimulatory molecules on DC, leading to an impaired

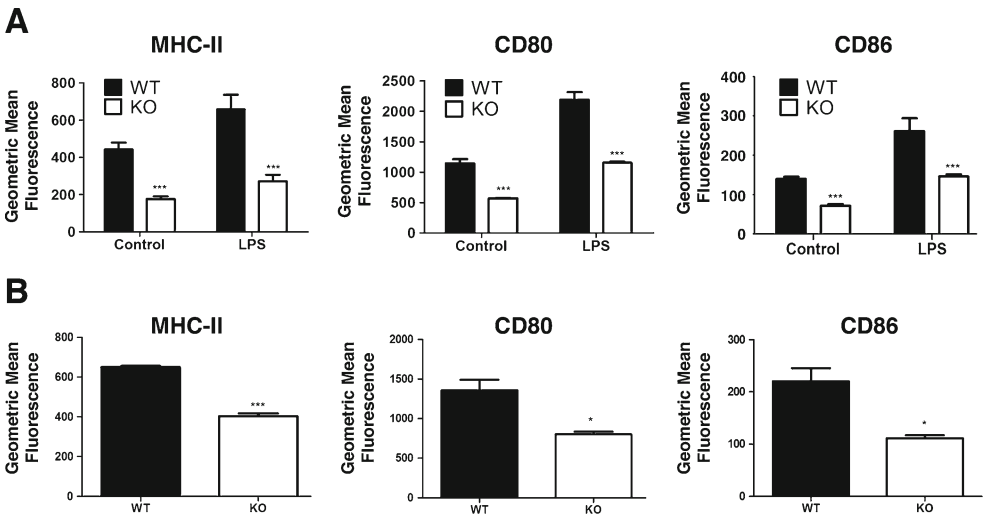

Fig. 1 DC that lack HIF-1α express lower levels of MHC-II and costimulatory molecules. Immature BM-derived DC were stimulated with 100 ng/ml LPS (**a**) or matured with 500 U/ml TNF-α (**b**) overnight and were evaluated for MHC-II, CD80, and CD86 expression using specific antibodies and FACS analysis. Statistical significance was calculated using (**a**) two-way ANOVA and (**b**) unpaired, two-tailed *t* test; $*P<0.05$, $***P<0.001$

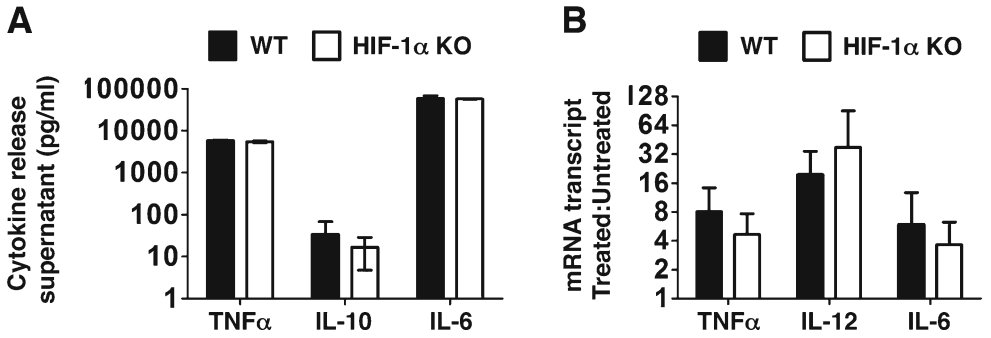

Fig. 2 HIF-1α is not essential for release of key cytokines from DC. (**a**) BM-derived DC from WT and HIF-1α myeloid null mice were stimulated with LPS (10 ng/ml) overnight; cytokine release was quantified by ELISA. Data shown are the result af an experiment with triplicate samples and representative of five experiments with similar results. (**b**) DC were stimulated with LPS (10 ng/ml) for 4 h and cytokine transcripts monitored by real-time RT-PCR. Data shown are the pooled results of five experiments. Statistical significance was calculated using two-tailed *t* tests

ability to stimulate both $CD4^+$ and $CD8^+$ T cell proliferation, without significant effects upon the release of cytokines. These changes correlate to a weaker cell-mediated and humoral response to vaccination. Elevating HIF with the drug AKB-4924 produces the opposite effects, enhancing DC antigen presentation and T cell stimulatory functions.

Our results provide new information relevant to a somewhat confounding literature regarding the influence of reduced oxygen tension on DC activity. Using murine [19] or human [30] DC, two groups separately found that while hypoxia alone did not affect antigen presentation or T cell activation, it did synergize with LPS to create a stronger

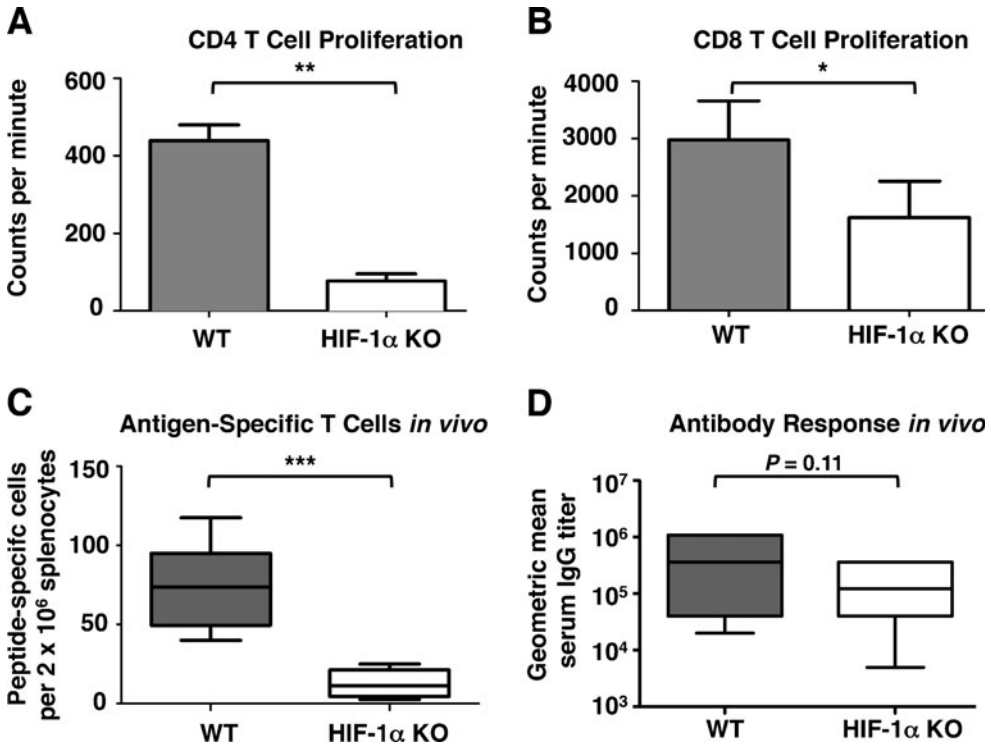

Fig. 3 HIF-1α is important for DC-triggered T cell proliferation, and HIF-1α myeloid-KO mice have weakened responses to vaccination. BM-derived DC were coated with the MHC-II-restricted OVA subpeptide $OVA_{323–329}$ and incubated with $CD4^+$ T cells from OT-II mice (**a**) or coated with the MHC-I restricted OVA subpeptide SIINFEKL and incubated with $CD8^+$ T cells from OT-I mice (**b**) for 3 days. T cell proliferation was measured by the incorporation of ^3H thymidine. Data represent the normalized results of three (**a**) or four (**b**) experiments. (**c**) Six myeloid HIF-1α/Tie-2 Cre^+ and six Cre^- littermate controls were vaccinated with 50 μg/mouse SIINFEKL peptide. Eight days after vaccination, mice were sacrificed, and specific T cells in their spleens measured by IFNγ ELISPOT. **d** Myeloid HIF-1α/Tie-2 Cre^+ and Cre^- mice were vaccinated with 50 μg/mouse OVA protein. Blood was collected 14 days after vaccination, and OVA titer was measured using a specific ELISA. Data shown are the combined results of two separate experiments, for a total of 11 WT and 9 KO mice. Statistical significance was calculated by two-tailed *t* test. $*P<0.05$, $**P<0.01$, $***P<0.001$

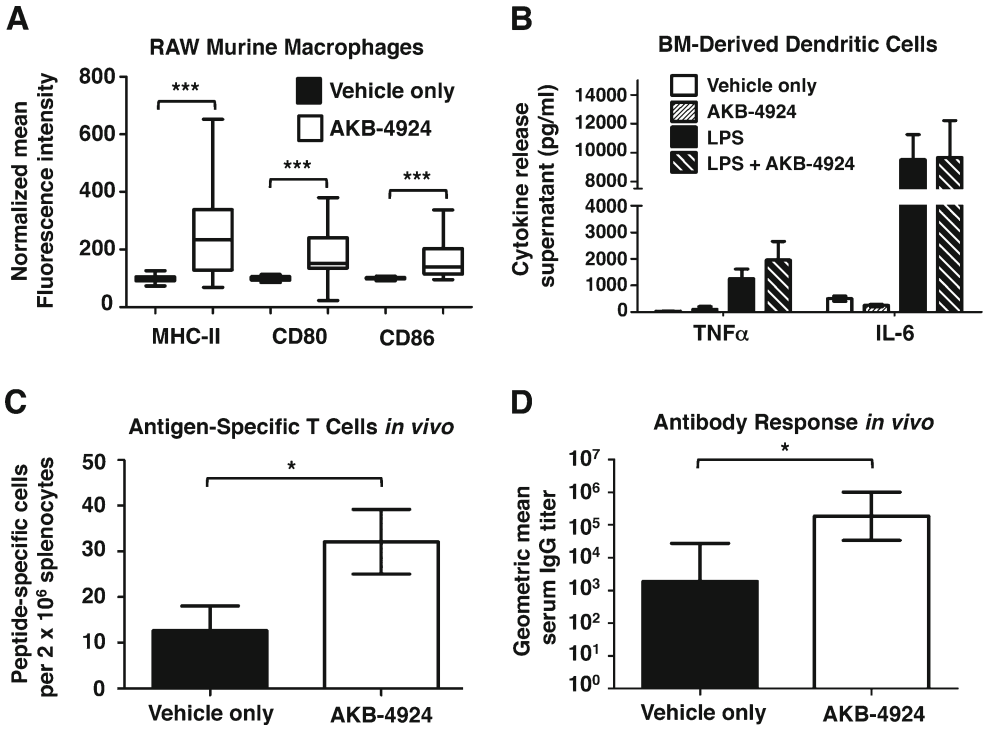

Fig. 4 Pharmacological augmentation of HIF-1α increases DC activation and response to vaccination. (**a**) RAW murine macrophages were treated with 100 μM AKB-4924 or vehicle (cyclodextrin) control overnight and surface marker expression measured by flow cytometry. (**b**) WT BM-derived DC were treated with 100 μM AKB-4924, 10 ng/ml LPS, both AKB-4924 and LPS, or vehicle control, and cytokine release measured by ELISA. (**c**) and (**d**) WT C57BL/6 mice (five per group) were pretreated with AKB-4924 (5 mg/kg intraperitoneal) or vehicle control before immunization with 50 μg of (**c**) SIINFEKL peptide or (**d**) OVA protein. (**c**) Splenic T cell response was measured by IFNγ ELISPOT 8 days after immunization; (**d**) anti-OVA serum IgG was measured by ELISA 14 days after immunization. Statistical significance was determined by two-tailed *t* test; **P*<0.05, ****P*<0.001

effect than LPS alone. Along those lines, it was reported that hypoxic macrophages were better at activating T cells while releasing more of the Th1-polarizing cytokine IL-12 [31]; however, other groups arrived at opposite conclusions, suggesting hypoxic DC had impaired antigen presentation and T cell activation [32] and promoted a Th2 phenotype [33]. Still others have reported a mixed phenotype [16]. Differences in experimental approaches used with regard to source and purity of DC precursors, differentiation/maturation protocols, and degree and duration of the hypoxic stimulus have been suggested as potential explanations for these discrepancies [15]. Our research was the first to couple genetically modified mice and pharmacological tools to distinguish the effect of the transcription factor HIF-1α specifically from more global, hypoxia-dependent effects.

The compound AKB-4924 has been found to stabilize HIF-1α activity in macrophages and neutrophils and to promote their direct bactericidal activity against drug-resistant pathogens [28]. This project was motivated in part by an interest in determining whether a similar approach to pharmacologically augment HIF-1α could be contemplated as an adjuvant strategy for vaccines. Drugs related to AKB-4924 that modulate prolyl hydroxylases have advanced in clinical trials of extended therapy to boost erythropoietin levels in anemia, showing a favorable safety profile to date [34]; thus their short-term use in vaccines would likewise be feasible. Our preliminary results show that pharmacologically increasing HIF-1 levels has a modest effect to improve the response to experimental vaccination. Whether this effect could be harnessed and optimized to prove useful in adjuvant formulations merits further exploration.

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Conflict of interest Randall Johnson, who provided the KO mice, is on SAB of Aerpio Therapeutics, who provided AKB-4924 HIF agonist. Victor Nizet and Aerpio Therapeutics have collaborated on NIH and Department of Defense grants. Aerpio Therapeutics had no interest in the design or reporting of the present study.

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