# Cell Culture Oxygen and Signaling: Supraphysiologic in vitro oxygen signals through the HIF-CXCR4 pathway in human B cells

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## Abstract

**Current understanding of B lymphocyte function relies heavily upon *in vitro* assays. However, standard $CO_2$ incubators maintain cells at supraphysiologic oxygen (16-19% $O_2$). Physiologic oxygen is far lower (blood 5-10%, secondary lymphoid tissues 1-5%, regions of inflammation, tumor, and bone marrow 0-1%). Previously, we showed that transcripts for hypoxia-induced factor 1 alpha (HIF-1a), an oxygen-sensing transcription factor, are upregulated in peripheral B cells after vaccination[1]. HIF-1a is also a therapeutic target in multiple myeloma[2]. Downstream of HIF-1a are genes tied to metabolism, proliferation, and the unfolded protein response. One gene downstream of HIF-1a that is critical for B cell migration and function is CXCR4. We set out to determine if room air incubation might affect B cells *in vitro,* our null hypothesis being that physiologic oxygen would not affect B cell HIF-1a or CXCR4 function. We used C-chamber incubator sub-chambers and Pro-Ox 110 gas controllers to control the incubation $O_2$ and $CO_2$ levels. Setting the reference frame to a range relevant to secondary lymphoid organs (1-5% $O_2$), we set the chambers to 5% $CO_2$ and 1% (hypoxic), 4% (physioxic), or 19% (supra-physioxic) $O_2$. In primary human B cells and myeloma cell lines, HIF-1a protein levels stabilized at 24hrs of culture at 1% and 4% $O_2$, but not at 19% $O_2$. More importantly, B cell migration in response to the CXCR4 ligand, CXCL12, was significantly decreased at low oxygen levels, without affecting cell proliferation or viability, disproving our null hypothesis. This has widespread implications for assessments of B cell function in that incubator oxygen drives HIF-1a degradation and CXCR4 responsiveness *in vitro*.**

## Background

- Immunometabolism is a new field in which the broad effects of cellular environment and differentiation/activation states upon lymphocyte function are being newly connected through oxygen and cell metabolic pathways
- HIF-1a/ mTOR pathways interact with NF-kB and GAPDH to alter lymphocyte function and lymphoma development[4].
- CXCL12 chemokine production by endothelial cells is directly related to the local oxygen level through HIF-1a levels[3], as is the expression of its receptor CXCR4.

## Objectives

**Assess primary B cell and B tumor cell line HIF-1a levels and CXCR4 function at different oxygen levels:**

**1% Hypoxic or Low Physioxic**
**4% Physioxic**
**19% Supraphysioxic (Traditional Room Air Culture)**

## Experimental Design

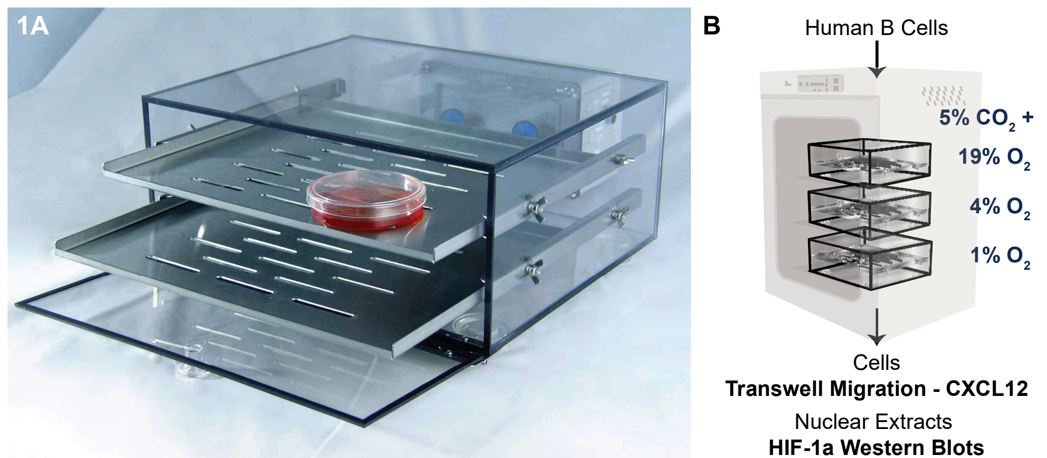
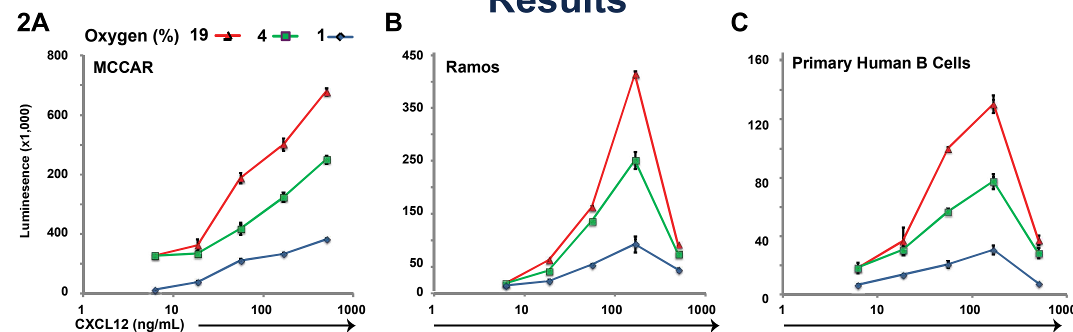



**Figure 1. Experimental Design. (A)** Individual C-Chambers incubation subchambers with Pro-Ox controllers were used to provide a controlled oxygen enviroment for each cell culture. (B) Cells were incubated in 5% $CO_2$ plus $O_2$ at 1%, 4%, or 19% (room air incubator oxygen). All media were pre-equilibrated to the proper oxygen level before use. Cells were put into a standard chemotaxis assays with a chemiluminescent read-out or cell lysates were subjected to HIF-1a western blot.

## Results



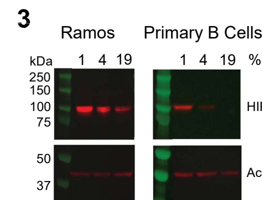




**Figure 2. Modulation of B Cell Migration by In Vitro Oxygen Levels.** Cell lines, MC-CAR (A) and Ramos (B), or primary human B cells (C) were incubated at 1%, 4%, or 19% $O_2$ in addition to 5% $CO_2$ overnight. MCCAR and Ramos cell lines (ATCC, USA) were incubated in DMEM and RPMI 1640 (respectively) plus 10% FBS (Gibco, USA) overnight. Primary B cells were isolated from fresh human blood by positive immunoselection (Miltenyi, USA) and incubated in RPMI 1640 +10% FBS. The next day, cells were added in triplicate to upper chambers of 96-well PC 5 um transwell plates (Corning). Media with the indicated human CXCL12 (R&D Systems) were added to lower chambers and plates incubated for 1 hr. CellTiter-Glo (Promega) and a Bio-Tek Synergy HT plate reader were used to detect the cells that in the lower chamber. An $O_2$-dependent effect was seen, with B cells migrating less in lower $O_2$. This is consistent with B cells migrating to and remaining in the low-oxygen environment of bone marrow. There were no differences in cell viability between the cultures.

**Figure 3. Oxygen-dependent HIF-1a Protein Stabilization in B Cells.** Nuclear extracts were prepared from the same cells as in Figure 2 using NE-PER (Life Technologies). Western blot anlysis was performed using a mu-anti human HIF-1a(R&D Systems) and detected with HRP- gt anti-mu 2º (BD) and ECL substrate (BioRad). This is consistent with model of B cell response to the increased $pO_2$ outside of bone marrow in peripheral circulation by modulation of HIF-1a and increased homing back to lower oxygen microenvironments.

## Conclusions

**There is a reciprocal association between *in vitro* $O_2$-dependent HIF-1a levels and B cell migration**
**This is consistent with B cells migrating to and remaining in bone marrow in response to environmental oxygen levels**
**Room air $O_2$ incubation may produce results that do not reflect B cell function *in vivo***
**Supraphysiologic $O_2$ effects may not be observed if only monitoring cells for viability**

## References

1 Henn, A. D. et al. High-resolution temporal response patterns to influenza vaccine reveal a distinct human plasma cell gene signature. Scientific reports 3, 2327, doi:10.1038/srep02327 (2013).
2 Storti, P. et al. Hypoxia-inducible factor (HIF)-1alpha suppression in myeloma cells blocks tumoral growth in vivo inhibiting angiogenesis and bone destruction. Leukemia 27, 1697-1706, doi:10.1038/leu.2013.24 (2013).
3 Ceradini, D. J. et al. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nature medicine 10, 858-864, doi:http://www.nature.com/nm/journal/v10/n8/suppinfo/nm1075_S1.html (2004).
4 Chiche, J. et al. GAPDH enhances the aggressiveness and the vascularization of non-Hodgkin's B lymphomas via NF-kappaB-dependent induction of HIF-1alpha. Leukemia 29, 1163-1176, doi:10.1038/leu.2014.324 (2015).