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Short Communication

The Role Of IL-16 as a lymphocyte attractant appears to be conserved through phylogeny: preliminary evidence that recombinant human IL-16 preferentially attracts regulatory lymphocytes in the amphibian, *Xenopus laevis*

Acadia L. Kopec, Zak E. Michaud, and Gregory D. Maniero*

[1]Stonehill College, Biology Department, 320 Washington Street, North Easton, MA 02357, USA

*Author for correspondence: Email: gmaniero@stonehill.edu

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Abstract

IL-16 is a pleiotropic, pro-inflammatory cytokine that induces regulatory $CD4^+$ T cells to migrate to a site of inflammation or tissue damage. IL-16 is a ligand for CD4 and binds at the proximal, D4 region well outside of the binding site for MHC class II. The sequence and structure of IL-16 is highly conserved among disparate vertebrates but CD4 is less well conserved and is highly variable at the distal, D1 region that binds to MHC class II during T-cell activation and effector function. Conversely, the D4 region, like its ligand, is very highly conserved. This conservation of sequence and structure suggests that the role of CD4 as a receptor for IL-16 has been retained throughout vertebrate phylogeny. Because of the conservation of this receptor: ligand pair, we set out to demonstrate that recombinant human IL-16 (rhIL-16) can elicit the same effects on lymphocytes from the amphibian *Xenopus laevis* as it would on human lymphocytes. Our data suggest that rhIL-16 attracts a population of $CD4^+$ lymphocytes with a regulatory phenotype.

Keywords: CD4, IL-16, CTLA-4, CD28, *Xenopus*

Introduction

Important biological functions are highly conserved and found, relatively unchanged, throughout phylogeny. The adaptive immune system as we understand it appeared with the advent of the jawed vertebrates and many of its components and functions are shared among disparate vertebrate groups. Our lab works with an amphibian model organism, the African clawed frog, *Xenopus laevis*, that occupies an interesting position in vertebrate phylogeny being an anamniotic tetrapod. The *Xenopus* immune system has both cellular and humoral components that are similar those of mammals, making this an excellent model for the study of essential components of vertebrate immune protection. The immune system of this anuran amphibian has been well characterized and studied for decades [1-3]. The overarching goal of our research is to describe an ancestral role for CD4 as a receptor for the cytokine IL-16 by examining the effects of recombinant human IL-16 (rhIL-16) on *Xenopus* lymphocytes.

IL-16 was initially characterized by its ability to attract lymphocytes in delayed-type hypersensitivity responses [4-8]. Originally named Lymphocyte Chemoattraction Factor (LCF), IL-16 has since been described as a pleiotropic cytokine primarily produced by $CD8^+$ T lymphocytes and endothelial tissue as well as some cells of the myeloid lineage, and its production can be stimulated by mitogens, histamines, and antigens [6-13]. The sequence and structure IL-16 are

highly conserved among disparate phylogenetic groups [9,14,15]. IL-16 is released following post-translational cleavage of a 68-kDa pro-IL-16 molecule and is secreted as either a monomer or as a more physiologically active tetramer [13]. In its active form, IL-16 is a 17kDa protein containing a PDZ domain that is partially blocked and unable to bind proteins [16]. Within this non-functional domain, there is a highly conserved motif (GLGF) that appears to be involved in tetramerization [6,16-18].

The receptor for IL-16 is the CD4-coreceptor of the T-cell receptor complex. The canonical role of CD4 is binding to MHC class II during helper T-cell (Th) activation and effector function. The distal, D1 domain of CD4 binds to highly conserved regions of the MHC class IIβ chain [19] to stabilize the MHC class II: TCR binding and to enhance intracellular signaling through CD4's association with the intracellular kinase $p56^{lck}$ [20-22]. The MHC class II-binding region of CD4 is highly conserved in mammals with an FLXK motif that has been found on all eutherian mammalian species thus far studied [23]. Although the D1 region of CD4 is highly conserved in mammals, this is not the case throughout vertebrate lineages. Although all gnathostomes show concrete evidence of helper T cell activity, the D1 region pf CD4 varies widely between disparate vertebrate groups [14,23]. In contrast, the intracellular region of CD4 that associates with $p56^{lck}$ appears to be highly conserved throughout vertebrate phylogeny [23].

IL-16 binds on the proximal D4 domain of CD4 an effectively long distance away from the MHC-binding site [6,16]. Because the CD4 D1 domain is highly conserved among mammals, human IL-16 recruits murine $CD4^+$ lymphocytes *in vitro* and mouse IL-16 similarly recruits human $CD4^+$ lymphocytes. In mice, IL-16-induced chemotaxis by $CD4^+$ lymphocytes, is blocked by addition of anti-human IL-16 antibodies [24].

We have shown previously that, although *Xenopus* demonstrates traditional Th effector functions, they do not possess the FLXK motif that is important for binding to MHC class IIβ and highly conserved in mammals. However, the highly conserved $p56^{lck}$-binding region is present in this amphibian [14,23]. Additionally, unlike the CD4 D1 domain, which varies significantly throughout vertebrate phylogeny, the D4 domain, particularly in the IL-16-binding region, is highly conserved from fishes to mammals, including in *Xenopus* [6,9,14,15].

In humans, IL-16 is produced both as a monomer and as an active tetramer. Native tetrameric human IL-16 and monomeric recombinant (rhIL-16) both bind to $CD4^+$ lymphocytes and induce migration [7,8]. $CD4^+CD8^-$, but not $CD4^-CD8^+$ cells migrate in response to IL-16, indicating that attraction to IL-16 is CD4-dependent [11,13,25]. In fact, IL-16 induces chemoattraction in direct proportion to the density of CD4 on the target cell surface [13]. Furthermore, Th1 cells are attracted to IL-16 to a greater extent than Th2 cells [25]. In addition to its role attracting lymphocytes, IL-16 binding to CD4 interferes with mixed lymphocyte reactions by preventing the interaction of CD4 with the remainder of the T-cell receptor complex [11]. IL-16 binding to CD4 increases the expression of various molecules on the surface of resting lymphocytes, including MHC class II and the receptor for IL-2 (IL-2R) [5,10,13,24].

We have shown that the predicted amino acid sequence of IL-16 from *Xenopus* is highly similar to that of human, mouse, rat, chicken, and trout IL-16 [14]. All of the amino acid motifs important to binding to CD4 are identical in all of these animals. This similarity, combined with the high degree of conservation of the CD4 D4 region, suggests that IL-16:CD4 binding should be highly promiscuous throughout vertebrate phylogeny. Our lab works under the hypothesis that human IL-16 will bind to *Xenopus* CD4 to attract and activate the amphibian lymphocytes. Although the *Xenopus* immune system is well characterized, there are no available antibodies that recognize Xenopus CD4 [23]. One of the goals of our work is to gain the ability to recognize *Xenopus* $CD4^+$ lymphocytes by their ability to bind IL-16. Although our lab is currently developing and verifying a recombinant *Xenopus* IL-16 fragment, the conserved sequences and structures of IL-16 and CD4D4 suggest that we should be able to utilize recombinant human IL-16 to recognize and stimulate *Xenopus* lymphocytes.

We have demonstrated the ability to separate a significant portion of *Xenopus* lymphocytes by incubation with rhIL-16 and separation on a magnetic column. We have also demonstrated that an injection of rhIL-16 into the *Xenopus* peritoneum results in an increase in lymphocytes in the peritoneal lavage that is significantly elevated above vehicle control and heat-killed bacteria injections. Our data also suggest that incubation with rhIL-16 results in the upregulation of MHC class II mRNA in $CD8^-$ lymphocytes preferentially over that of $CD8^+$ lymphocytes [14].

Due to its ability to induce lymphocyte migration, IL-16 is classified as a pro-inflammatory cytokine yet it appears to slow TCR-mediated activation [5]. As a chemoattractant of $CD4^+$ lymphocytes, IL-16 appears to preferentially attract and activate regulatory T cells (Tregs), which suppress T cell activity. IL-16 inhibits the production of IL-2 by mitogen activated $CD4^+$ lymphocytes in humans and preferentially attracts lymphocytes that express mRNA for FoxP3 *in vitro* [26]. During inflammatory lung injury, IL-16 produced in part by the lung endothelium, attracts $CD4^+$ T cells that express FoxP3 and produce IL-10 and appear to protect the lungs from infiltration by neutrophils [27]. T cells that migrate *in vitro* in response to IL-16 in trans well experiments express more CD25 and CTLA-4 on their surface and release more TGFβ than control cells. In addition, cells that migrate in response to IL-16 express higher levels of FoxP3 mRNA and protein than do control cells [28], suggesting that IL-16 attracts primarily T cells with a regulatory phenotype.

We hypothesize that rhIL-16 affects the migration of *Xenopus* lymphocytes as it does human cells. To test this hypothesis, we continue to characterize the cells that migrate to the *Xenopus* coelom following an injection with rhIL-16. The purpose of this paper is to follow up on our earlier work with in vivo chemoattraction and begin to characterize the *Xenopus* cells that are attracted in this assay, with particular attention to potential markers of regulatory phenotype.

Methods

An *in vivo* cell migration experiment was carried out as previously described [13] using outbred, adult *Xenopus laevis* (*Xenopus* 1, Dexter MI). All animal procedures were approved by the Institutional Animal Care and Use Committee of Stonehill College. Briefly, 200 µL of either Amphibian Phosphate Buffered Saline (APBS, 80% dilution of PBS, Sigma Aldrich, St. Louis, MO), a suspension of heat-killed *E. coli*, or 10 nM rhIL-16 in APBS was injected into the peritoneum of each of three animals selected randomly for each

group. After 17-18h, the frogs were narcotized in 1.0 g/L tricaine methanosulfate (MS-222, Sigma Aldrich) until unresponsive. The peritoneal cavity of each frog was lavaged with approximately 7 ml of APBS, the body was gently massaged to mix, and as much fluid as possible was recovered. Peritoneal exudates were centrifuged at 2000 rpm, resuspended in 1 mL of fresh APBS, and transferred to a microcentrifuge tube. Each sample was centrifuged at 14,000 rpm for 5 minutes, the supernatant removed and the cell pellet resuspended in 750 µL of Trizol reagent (Life Technologies, Carlsbad, CA) and mRNA was collected following the manufacturer's protocol. Approximately 500ng of mRNA was reverse transcribed to cDNA using an iScript (Bio-Rad, Hercules, CA) kit and polymerase chain reaction was performed using One Taq RT-PCR (New England Biolabs, Ipswich, MA). Amplification was performed using 2µM primers specific for *Xenopus laevis* β-actin, CD4α, CD8, CD8β, MHC class IIα, and MHC Class IIβ (Table 1, sequences from [3]) as well as primers for CD28, CTLA-4, IL-10, and FoxP3 (Table 1). All reactions were carried out with 35 cycles of 30 seconds at 95°C, 30 seconds at a fragment-specific annealing temperature (Table 1), and 30 seconds at 68°C. The amplified fragments were visualized by electrophoresis on 2% agarose gels.

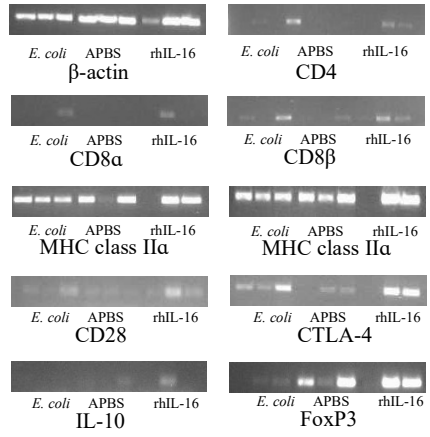

Figure 1: 2% gel electrophoresis of RT-PCR products of cDNA from peritoneal samples after injection with heat-killed E. coli, Amphibian Phosphate Buffered Saline (APBS), or recombinant human IL-16 (rhIL-16).

Fragment	Forward Primer (5′ – 3′)	Annealing Temperature (°C)	Fragment Length (bp)
B-actin*	GGTGTCATGGTTGGAATGG TGTGGTTACACCATCACCTG	58.3	359
CD4*	TCCATCTCTGACATCCCCTC TCACCAGACACACGTCCATT	60.0	895
CD8 α chain*	AAGCCACCTACGACTACCACCAA CCGTTCTTCTCAGTCTCAGGCAC	59.0	247
CD8 β chain*	TCATCATCTCTTTCTGGGGC ZZTTCAGTGGGTGCTTCCTG	60.0	604
MHC Class II α chain*	TCAAAGTCAGTGGTTTGGACG GTGTAAATATCATGTTCATTTG	54.0	390
MHC Class II β chain*	ACGGCACCGACAATGTCAGG TTAATGGGTGTCTCCAGCATC	61.0	450
CD28	TCCATAAAGGGCCTTGGCAAT CAATGGCAAAATAGGCTGTGGT	60.0	210

CTLA-4	TGTGCCTAATCACCAATGCCT TGTCAGGTGTCTGTAGCCCT	60.0	288
IL-10	GAGATGTCAAAGCGGAGGGG TTTCAGACACGGACTGGCAT	57.5	197
Fox-P3	AAATCAACCCTTGGAGCCGT GCCCAACCCAGGCTAGTAAG	57.0	367

Table 1: PCR primers, annealing temperatures and fragment length (* Chida et al. [23]).

Results and Discussion

We have demonstrated previously [13] that a single injection of rhIL-16 in the peritoneum of *Xenopus laevis* will result in the accumulation of lymphocytes in the body cavity. We have repeated this experiment and, upon visual inspection, we see once again that lymphocytes migrated in response to rhIL-16 but not vehicle or bacteria controls. Here we sought to characterize the recruited cells through gene-expression analysis. Due to the lack of anti-*Xenopus*¬ CD4 antibodies, cells cannot be identified by flow cytometry or fluorescent microscopy. We instead chose to characterize these cells by analysis of mRNA expression. We hypothesized that cells that migrated in response to rhIL-16 would be primarily $CD4^+$ T lymphocytes with a predominantly regulatory or immunosuppressive phenotype. Figure 1 suggested that chemoattraction to rhIL-16 by peritoneal lavage cells correlated with expression of CD4 mRNA more than that of CD8α or β. As expected, cells from both experimental and control animals expressed MHC class II (as do all *Xenopus* lymphocytes) although both the MHC class II α and β chain were most highly expressed in cells that are attracted to IL-16. Our prior study suggests that incubation with rhIL-16 initiated expression of MHC class II α and β chains in $CD8^-$ lymphocytes to a greater amount than those that are $CD8^+$ and it is possible that the rhIL-16 not only acted as a chemoattractant but may have induced activation and upregulation of expression of some genes. Additionally, the current analysis of gene expression demonstrated that the cells that were recruited by rhIL-16 expressed mRNA for CTLA-4 to a greater extent than that of CD28, indicating that these lymphocytes exhibit a regulatory phenotype. In humans, IL-16 recruits $CTLA4^+FoxP3^+$ Treg cells that function to suppress Th2 cytokine production and regulate inflammation. Incubation with IL-16 also induces the expression of FoxP3 mRNA [28]. Our results suggest that rhIL-16 induced changes in lymphocytes from an amphibian; stimulating the migration of $CD4^+CTLA\text{-}4^+$ lymphocytes.

Our preliminary data also suggest that the *Xenopus* cells that migrated in response to rhIL-16 express mRNA for both IL-10 and FoxP3, further supporting our hypothesis that rhIL-16 lymphocytes with a regulatory phenotype. Once again, we cannot rule out the possibility that the exogenous rhIL-16 induced the expression of regulatory genes in the cells that were recruited to the *Xenopus* body cavity.

In conclusion, we have demonstrated that human IL-16 recruits T lymphocytes that express mRNA for CD4 not CD8, CTLA-4 to a greater extent than CD28 and IL-10, as well as FoxP3, suggesting that these are regulatory $CD4^+$ T cells. Human IL-16 acts as a chemoattractant for regulatory $CD4^+$ T cells *in vivo* and appears to recruit a similar subset of cells in an amphibian. This suggests that the receptor: ligand interaction of CD4 and IL-16 is a highly conserved and an important facet of vertebrate immunity.

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