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Chapter

Evolutionary Conservation of the Role of CD4 as a Receptor for Interleukin-16

Gregory D. Maniero

Abstract

The interaction of CD4 with MHC class II during helper T-cell activation and effector function is required for the initiation of an adaptive immune response in all gnathostomes. CD4 is comprised of four immunoglobulin domains but most likely arose from an ancestral two-domain homolog. The distal, D1 domain of CD4 binds to non-polymorphic regions of the MHC molecule, but despite the absolute requirement for this interaction, the sequence and structure of this domain are not well conserved through phylogeny. Conversely, the proximal, D4 domain of CD4 contains the binding site of the cytokine IL-16 and is highly conserved in its amino acid structure. IL-16 is a cytokine that has been described in a wide variety of invertebrate and vertebrate species. The CD4-binding residues on IL-16 are highly conserved throughout phylogeny, allowing for promiscuous binding of IL-16 to CD4 between members of unrelated taxa. This chapter aims to present structural, and functional support for the hypothesis that the CD4 co-receptor of the TCR arose from a primordial receptor for IL-16.

Keywords: IL-16, CD4, evolution, chemoattraction, T cells

1. Introduction

The importance of acquired immunity for the survival of an organism in the face of an environment full of potential pathogens cannot be understated. The immune functions essential to acquired immunity arose with the vertebrates [1–3] and rely heavily on the activation of a $CD4^+$ subset of T lymphocytes. The role of $CD4^+$ lymphocytes is well known and seems to have appeared with gnathostomes. Although T-helper (Th) cells require interactions between CD4 and classical MHC class II, both molecules most likely did not arise at the same point in evolution. A growing body of evidence suggests that CD4 was originally a receptor for Interleukin-16 (IL-16) that was integrated into the immune system as a co-receptor of the T-cell receptor (TCR) complex. The aim of this chapter is to present some of the structural and functional characteristics that have been retained in CD4 among diverse vertebrate taxa, and the same type of characteristics retained in IL-16 throughout phylogeny, including in species much older than vertebrates.

2. Il-16

IL-16 was first described in 1982 as Lymphocyte Chemoattraction Factor (LCF) for its ability to recruit lymphocytes independent of antigen specificity to the site of inflammation during a delayed-type hypersensitivity (DTH) reaction [4–8]. IL-16 is a unique [6, 9] pleiotropic cytokine that is secreted primarily by $CD8^+$ T cells but can also be produced by eosinophils, dendritic cells (DCs), monocytes, macrophages, mast cells, as well as activated $CD4^+$ T cells, fibroblasts, and bronchial epithelial cells [10–14]. Production of IL-16 can be stimulated by mitogens or histamines as well as by recognition of antigen [4–6, 9, 15]. IL-16 influences pathological states including asthma and multiple sclerosis. Additionally, IL-16 mRNA is often present in lymphoid organs, suggesting a role in normal immune function [11].

IL-16 is post-translationally cleaved by caspase 3 from the C-terminal end of a 68-kDa pro-IL-16 molecule [16–18]. Active IL-16 is a 17 kDa protein that contains a single PDZ domain [6, 7]. Unlike in other PDZ-containing proteins, this domain is not functional for protein binding as it is partially blocked by a nearby tryptophan side-chain [19]. Native IL-16 is released as a monomer and as a tetramer, which is the primary active form of the molecule [9]. A GLGF motif within the PDZ domain may be important for the oligomerization of IL-16 [6, 17–19] and secreted monomeric IL-16 will auto-aggregate to spontaneously form active tetramers [11]. IL-16 is stored in its active configuration in granules of $CD8^+$T cells that are the main contributors of this cytokine in an immune response [16–18]. Although tetrameric IL-16 is the primary active form, monomeric IL-16 is capable of binding to CD4 and can induce a variety of phenotypic changes in target cells [9, 16].

3. IL-16 as a ligand for CD4

IL-16, whether in monomeric or tetrameric configuration, most notably attracts $CD4^+$ cells and most IL-16-mediated cell migration has been demonstrated in human and murine lymphocytes. The only described receptor for IL-16 is the CD4 co-receptor of the TCR complex, and IL-16-induced lymphotaxis is strictly limited to $CD4^+$ cells [6, 14], as evidenced by the fact that $CD4^+$, but not $CD4^-$ T cells migrate in response to IL-16 [9, 20]. Additionally, the degree of IL-16-induced chemotaxis in vitro is proportional to the density of CD4 on the surface of the responding lymphocytes [9]. The chemoattractant activity of recombinant IL-16 (rIL-16) is blocked by preincubation with F_{ab} fragments of the anti-CD4 mAb OKT4 [9, 15]. Furthermore, IL-16 elicits the migration of Th1 lymphocytes more than that of Th2 cells [19]. Recombinant human IL-16 (rhIL-16) initiates T-cell chemotaxis, in vitro, at 10 nM rhIL-16 to as low as 0.001 nM with a 50% effective dose (ED_{50}) of 0.01 nM [6, 20, 21].

Upon binding to CD4, IL-16 induces a variety of changes on Th cells in addition to chemotaxis. In addition to being a growth factor for $CD4^+$ T cells, IL-16 synergizes with IL-2 to promote the expansion of T-cell populations [12]. The binding of CD4 by IL-16 stimulates T lymphocytes to upregulate the production of various secreted and surface-bound proteins. Following incubation with IL-16, $CD4^+$ lymphocytes increase IL-2R on the cell surface [7, 9, 12], which indicates that IL-16 is involved in the expansion of T-cell populations independent of the recognition of antigen on MHC. Both native and recombinant IL-16 can induce expression of classical MHC class II molecules (primarily HLA-DR) on the membrane of non-stimulated human $CD4^+$ T cells in vitro [7, 9, 16] as well as stimulate them to produce GM-CSF [12]. Incubation with rhIL-16 will cause conA-stimulated, human $CD4^+$ MHC class II^+ PBMCs to down-regulate production of IL-2 [22]. By interfering with

the interaction of CD4 with the TCR complex, rIL-16 effectively disrupts mixed lymphocyte reactions (MLR, [15]). Attraction of $CD4^+$ Th cells is induced by both native IL-16 and monomeric rIL-16 [4, 5].

4. IL-16 is an ancient and ubiquitous cytokine

IL-16 is a cytokine that is found ubiquitously throughout vertebrate phylogeny and has been described not only in humans but in a variety of mammals [20], birds [23–26], fish [27, 28]. IL-16 has even been described in invertebrates [29–31] Additionally, inferred protein sequences from genetic data can be found for IL-16 and pro-IL16 in amphibians and reptiles [32]. The sequence and structure of IL-16 and pro-IL-16 show considerable homology among disparate vertebrate groups [13, 32, 33]. In mammals, IL-16 is highly conserved among humans, mice, and African green monkeys at the structural and genetic levels [6, 16]. Conserved residues on IL-16 that have been determined to be important for binding to CD4 by competitive binding assays are arginines at positions equivalent to human 106 and 107, and possibly a leucine at the equivalent of position 108 [13, 19].

5. CD4 as a receptor for IL-16

Although this chapter focuses on its functions as receptor for IL-16, CD4 is predominantly known for its role as a co-receptor in the T-cell receptor complex. As a co-receptor, CD4 binds, in conjunction with the α:β TCR, to MHC class II during antigen-dependent helper T-cell (Th) activation, differentiation, and effector function to substantially increase TCR-signaling [34, 35]. CD4 is a non-polymorphic 55-kDa monomer that consists of four extramembrane, Ig-like regions named from the distal D1 to the proximal D4 region [17, 36–39]. The D1 and D3 regions closely resemble V-type immunoglobulin domains, and D2 and D4 resemble C-type domains [34, 38, 40]. The CD4 co-receptor is expressed on the surface of Th cells and on some subsets of neutrophils and monocytes. Several models have been proposed to explain the association between CD4 and the α:β TCR on Th cells however the functional result of all of them is that CD4 associates with the TCR and enhances effector function [41]. The distal D1 region of CD4 interacts with the non-polymorphic region on the β2 domain of MHC class II molecules [39, 42]. This CD4:MHC association appears to stabilize and increase the affinity of TCR binding to antigen-bearing MHC class II molecules expressed on the surface of antigen presenting cells (APC) [42–44]. Additionally, CD4 augments intracellular signaling by recruiting the intracellular kinase $p56^{lck}$ that enhances the phosphorylation of ITAMs (immunoreceptor tyrosine-based activation motifs) upon the engagement of a TCR with its cognate antigen:MHC molecular complex. The phosphorylated ITAMs recruit ZAP70 that, when phosphorylated by $p56^{lck}$, continues downstream T-cell activation events [45]. The inclusion of CD4 in the immune synapse is necessary for effective T-cell effector function mediated by signaling through the TCR complex [34, 37].

A typical Th response begins with the engagement of the TCR with its cognate antigen:MHC complex. Complete cellular activation requires interaction with the CD4 co-receptor, the CD3 tetramer, and the intracellular ζ-chain. Binding of CD4 without subsequent co-receptor signaling can result in incomplete T-cell activation, limited IL-2-mediated proliferation, and eventual anergy. Crosslinking of the CD4 co-receptor results in downstream signaling that is independent of antigen, the TCR, and CD3. Partial activation can occur with cross-linking of CD4 by anti-CD4

$F(ab')_2$ fragments in the absence of antigen recognition or cell-to-cell contact. As in normal $CD4^+$ T-cell signaling, the low-level stimulation of exclusively CD4 engagement is due to the recruitment and activation of $p56^{lck}$ [10]. Phosphorylation and activation of $p56^{lck}$ initiates cell migration and increases in intracellular NFκB, IP_3, and Ca^{2+} as well as nuclear translocation of PKC [6, 10, 12, 15]. Treating $CD4^+$ immune cells with IL-16 results in a cell phenotype that bears a striking resemblance to that seen following anti-CD4 treatment [6].

Cross-linking of CD4 by tetrameric IL-16 or binding by monomeric protein results in the association of CD4 with, and the phosphorylation of $p56^{lck}$ [9, 10]. Transfection with human CD4 allows murine hybridomas to migrate in response to IL-16, but this response is absent in cells transfected with a mutant CD4 variant that is unable to associate with cytoplasmic $p56^{lck}$ [10, 20]. Native, but not recombinant, IL-16 stimulates $CD4^+$ T cell proliferation [7, 18], whereas rhIL-16 stimulates $CD4^+$ lymphocyte progression from G_0 to G_{1a} but does not initiate proliferation [6, 16, 22]. Both native and recombinant IL-16 will induce the expression of MHC class II (specifically HLA-DR) on the surface of resting human $CD4^+$ lymphocytes [7, 9, 42] and can induce their production of GM-CSF in vitro [12]. Since IL-16 can spontaneously form tetramers following release, it is difficult to tease out the difference between activation by monomeric and tetrameric IL-16. In addition to initiating signaling through CD4, IL-16 blocks the interaction of the CD4 co-receptor with the TCR complex. In fact, this is the mechanism that is responsible for IL-16-mediated disruption of in vitro MLR [15].

6. IL-16 preferentially recruits and activates regulatory T cells

In part 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 [7]. As a chemoattractant of $CD4^+$ lymphocytes, IL-16 appears to preferentially attract and activate regulatory T cells (Tregs), which suppress T cell activity [21]. 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 [22]. During inflammatory lung injury, IL-16 produced in part by the lung endothelium, attracts $CD4^+$ T cells that express FoxP3, produce IL-10, and act to protect the lungs from infiltration by neutrophils [46]. T cells that migrate in vitro in response to IL-16 in transwell 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 [21], suggesting that IL-16 primarily attracts T cells with a regulatory phenotype. Recombinant rhIL-16, as well as recombinant *Xenopus* IL-16 (rXIL-16, Maniero, unpublished data), recruits lymphocytes to the body cavity of *Xenopus laevis*. Upon examination, the recovered lymphocytes are seen to express mRNA for CD4 to a greater extent than for CD8, CTLA-4 more than CD28, as well as both FoxP3 and IL-10, suggesting a regulatory phenotype for the IL-16-recruited lymphocytes [47]. These mRNA levels were found in cells that were recovered approximately 16 h post injection with IL-16, so it is impossible to distinguish, from these experiments, if regulatory cells are attracted by IL-16 or if IL-16 induces the expression of a suite of regulatory genes [47].

7. Conservation of CD4

CD4 is highly conserved in mammals, yet the primary and secondary structures vary considerably among vertebrates [32, 38]. In the distal, D1 region, the canonical MHC class II-binding motif of FLXK is found on all eutherian mammals that have been

studied [32, 38]. However, even though all gnathostomes demonstrate CD4-dependent Th-activity, the D1 region is not highly conserved among representatives of disparate vertebrate groups [32, 38]. Most non-mammalian vertebrates do not possess the classic FLXK MHC class II-binding motif seen in mammals [32] yet rely heavily on traditional T-helper activity, suggesting that the role of CD4 binding to classical MHC molecules is an important function that has arisen on multiple occasions in vertebrate evolution. Other conserved motifs on the CD4 molecule are not involved with MHC class II binding. These conserved regions include a WXC motif and the intracellular CxC motif that associates with $p56^{lck}$ in the cytosol [34, 38]. The association of $p56^{lck}$ with CD4 is imperative for Th cell activation and the conservation of the lck-binding site demonstrates the essential and primordial association between these molecules [41, 48].

Although CD4 binds to ligands other than MHC class II molecules, including the surface glycoprotein gp120 of the Human Immunodeficiency Virus (HIV), which binds outside of the MHC class II-binding domain [34, 42, 43], the only described physiological role for the proximal, D4 region of CD4 is that of a receptor for the cytokine IL-16 [6, 19]. The IL-16 binding site on the proximal D4 domain of CD4 is an effectively long distance from the MHC-binding site [6, 19, 34]. On human CD4, there are two points on the D4 domain that are important for the binding of IL-16. A WQCLL motif from amino acids 344–348 is of major importance, but two valines at position 334 and 336 have been shown to be important in humans [33].

Amino acid sequence alignments, produced with CLUSTALW (www.genome.jp), show that the proximal D4domain of CD4, and especially the binding site for IL-16, is highly conserved (**Figure 1**), allowing for promiscuous binding of IL-16 to CD4 between disparate gnathostomes. In fact, human IL-16 recruits

Figure 1.
*Multiple alignment (CLUSTALW) of deduced amino acid partial sequences from CD4 D4 region of several vertebrates. The top portion presents the proximal, D4 domain of CD4. Conserved cysteines near the N-terminus of the proximal Ig domain are bolded and marked with an ▲. The canonical mouse and human WQCLLS motif that corresponds to human Val334, Val336, Gln345, is shaded, as are conserved residues that occupy the positions of and Leu347 and Leu347 required for binding IL-16 on human CD4 [19]. The bottom section shows amino acids from the transmembrane region and the cytoplasmic tail of CD4. The box surrounds the putative (CxC) binding site for p56lck . (human:*Homo sapiens *NCBI accession no. NP_000607.1, mouse:* Mus musculus *NP_038516.1, rat:* Rattus novegicus *NP_036837.1, chicken* Gallus gallus *CAA72740.1,* Xenopus laevis *NP_001233240.1, zebrafish CD4–1:* Danio rerio *XP_005173553.1, catfish CD4–1:* Ictalurus punctatus *NP_001187155.1, trout CD4:* Oncorhynchus mykiss *AAY42070.1, Salmon CD4-like* Salmo salar *XP_014019051.1, Fugo CD4–1;* Takifugu rubripes *NP_001072091.1, Tetraodon CD4–4b;* Tetraodon nigroviridis *ABU95654.1.*

murine $CD4^+$ lymphocytes in vitro, and mouse IL-16 similarly recruits human $CD4^+$ lymphocytes [16]. In mice, IL-16-induced chemotaxis of $CD4^+$ lymphocytes is blocked by the addition of anti-human IL-16 antibodies [16]. As would be expected with the conservation of the IL-16 binding region on CD4, IL-16 from derived vertebrates can activate $CD4^+$ T lymphocytes from more ancestral organisms [32]. Recombinant human IL-16 (rhIL-16) binds to lymphocytes from the African Clawed Frog, *Xenopus laevis* with sufficient avidity to allow rhIL16-bound lymphocytes to be separated on magnetic columns [32]. No reagents exist that can positively identify CD4 on *Xenopus* cells [3], and magnetic bead separation can merely suggest that rhIL-16 is binding to *Xenopus* CD4. Monoclonal antibodies specific for *Xenopus* CD8, however are available and can be used to isolate *Xenopus* $CD8^+$ T cells [3]. Incubation of *Xenopus* lymphocytes with rhIL-16 in vitro, correlates with the expression of MHC class II mRNA by $CD8^-$ cells but not those that are $CD8^+$, indicating that rhIL-16 is most likely binding to *Xenopus* $CD4^+$ lymphocytes [32]. As explained earlier, the role originally attributed to IL-16 was lymphocyte attraction, and injection of rhIL-16 into the body cavity of the amphibian *Xenopus* leads to the accumulation of lymphocytes in the peritoneum [32, 47]. The cells that are recruited to the *Xenopus* body cavity by rhIL-16 express mRNA for CD4 to a greater extent than that for CD8α or CD8β [32], again suggesting that rhIL-16 is recruiting $CD4^+$ *Xenopus* lymphocytes. The ability of IL-16 to affect CD4 cells from members of disparate vertebrate groups is hardly surprising. Not only is the IL-16-binding site highly conserved on CD4 (**Figure 1**), but the region of IL-16 that binds to CD4 is highly conserved throughout phylogeny (**Figure 2**).

Figure 2.
*Multiple alignment (CLUSTALW) of IL-16 deduced amino acids from several different vertebrates. The conserved GLGF binding cleft of the PDZ domain highlighted in gray. The residues that are critical for binding to domain 4 of CD4 to initiate chemotaxis, Argenines106–107 and Lysine108, are conserved throughout phylogeny and are underlined and bolded. (human:*Homo sapiens *NCBI accession no. AAC12732.1, mouse:* Mus musculus *AAC16039.1, rat:* Rattus novegicus *XP_006229550.1, chicken:* Gallus gallus *NP_001264925.1,* Xenopus laevis *XP_018108634.1, rainbow trout:* Oncorhynchus mykiss *CAD70074.2, puffer fish:* Tetraodon nigroviridis *AAX36076.3, shrimp:* Penaeus vannamei *ASJ26360.1, mitten crab:* Eriocheir sinensis*, Mud crab: (Gu et al., 2017), leech:* Hirudo medicinalis *ACF07997.1.*

8. The binding domains for IL-16:CD4 interactions are highly conserved

IL-16 is a cytokine that is produced by many organisms. Two residues crucial for binding to CD4 and homologous to human $arg^{106–107}$, are highly conserved in IL-16 from mammals, birds, amphibians, and teleost fish (**Figure 2**) [13, 19, 32]. Additionally, the GLF cleft of the IL-16 PDZ domain necessary for IL-16 oligomerization (**Figure 2**) [6, 16, 18, 19] is highly conserved throughout phylogeny [32]. The conservation of the D4 region of vertebrate CD4, along with the conservation of vertebrate IL-16, especially the conserved arginine residues that bind to CD4, certainly explains the intraspecies promiscuity of the IL-16:CD4 binding and activation [32].

In addition to vertebrates, IL-16 or proIL-16 has been described in several species of invertebrates, including the Chinese Mitten Crab *Eriocheir sinensis,* [49]), the mud crab (*Scylla paramosain*, [29]), and the Pacific white shrimp (*Litopenaeus vannamei*, [30]). A homolog of IL-16 has even been described from the nervous system of the European medicinal leech, *Hirudo medicinalis* [31]. The amino acid sequences of invertebrate IL-16 homologs indicate that these molecules contain the conserved arginine residues necessary for interaction with CD4 (**Figure 2**). These residues are equidistant from the PDZ domain in vertebrate IL-16 in all of the sequences that we examined. This is of particular importance since these organisms lack CD4, a molecule that is only found in vertebrates. The conservation of these residues on ancestral organisms has not been demonstrated previously, but strongly argues for the existence of a receptor for IL-16 that has at least some similarity to the D4 domain of vertebrate CD4.

Jawed vertebrates possess similar adaptive immune systems that rely on helper T-cell effector functions that depend, in a large part, upon CD4-MHC class II interactions. A vast majority of jawed vertebrates, with some notable exceptions, express CD4 on a subset of lymphocytes despite the fact that genes for CD4 are not well conserved among disparate species, even if they are closely related [50]. The gene for CD4 has been described and cloned in many species of teleost fish [51–58]. Helper, $CD4^+$ T cells in teleost function in a manner similar, if not identical to that found in their tetrapod counterparts. Like those in tetraposds, teleost T cells develop in the thymus as $CD4^+CD8^+$ double-positive cells migrate to the periphery as single positive lymphocytes. As in mammals, teleost $CD4^+$ T cells proliferate in mixed lymphocyte reactions and in an antigen-specific manner [55]. Helper T-cell function has been documented in fish, and adoptive transfer of $CD4^+$ cells from sensitized fish enhances virus-specific antibody formation [59].

9. Two-domain, CD4-like molecules

Two discrete forms of CD4 have been described in teleosts; one, consisting of four immunoglobulin-like domains that folds in a manner similar to that of tetrapod CD4 and interacts with classical MHC class II molecules [57], and a second type of CD4 molecule, that consists of only two immunoglobulin-like domains that does not appear to possess the ability to interact with MHC class II that is referred to as CD4–2, CD4REL, or CD4-like [50]. These two-domain CD4 molecules are expressed on the surface of a limited subset of teleost T cells and have cytoplasmic tails that associate with kinase $p56^{lck}$ like those of canonical, four-domain CD4 molecules [40, 50, 53, 58]. A genes for a four-domain CD4 molecule has been described in lamprey, but this lamprey CD4-like molecule does no include a canonical CXC motif that is required for the interaction with $p56^{lck}$ [60, 61]. Elasmobranchs lack

genes for either two- or four-domain CD4 molecules but possess genes for both MHC class I and MHC class IIα and β. Elasmobranchs exhibit only T-cell responses of a Th1 phenotype. Additionally, these primitive cartilaginous vertebrates possess CD4/LAG3-like genes that may encode an as-yet un-described molecule that is functionally homologous to CD4 in derived vertebrates [62, 63].

The two-domain, CD4-like molecules in fish have a proximal domain (D2) that possesses structural similarity to immunoglobulin constant regions (C-like) and a distal domain (D1) more similar to Ig variable domains (V-like) [61]. As stated above, the CD4 molecule of all gnathasomes, including teleosts, consist of four immunoglobulin-like domains. Due to the repeating domain structure of this molecule it has been postulated that the genes for typical CD4 molecules are derived from a duplication of an ancestral gene that encoded a two-Ig-domain, CD4-like, cell-surface molecule [64], although this does not explain the sole, four-domain CD4-homolog seen in the ancestral cyclostomes. In *Tetraodon,* lymphocytes that express CD4–2 appear to bind and migrate in response to recombinant IL-16 preferentially compared to those that express CD4–4 [56]. Additionally, the CD4–2^+ *Tetraodon* lymphocytes appear to have a regulatory phenotype, expressing mRNA for FoxP3 and having a CD25-like molecule on their surface [56]. The affinity of the likely ancestral CD4–2 for IL-16, and the possibility that IL-16 recruits CD4–2^+ regulatory lymphocytes supports our hypothesis that four-domain CD4 arose from an ancestral, two-domain interleukin receptor.

Many, and perhaps all four-domain CD4 molecules possess amino sequences that are homologous to human to IL-16-binding residues (**Figure 1**), and it seems that similar motifs are present on the proximal domains of two-domain CD4-related proteins. Although not identical to those seen on four-domain proteins, the deduced amino acid sequences of two-domain CD4 homologs from teleosts show potential IL-16-binding motifs that are spaced equidistant from the conserved cysteine at the N-terminal region of the most proximal Ig-like domain seen in traditional CD4 molecules (**Figure 3**). In all of the teleost two-domain CD4s that we have compiled, there is a valine at a position similar to the mammalian val^{336}. Additionally, two-domain CD4 homologs possess a sequence similar to the four-domain WQCLL motif, again, in a similar, if not identical, position in the proximal Ig-like domain. Rather than WQCLL, the two-domain motif is WTCQ(or L, K, or T)I (or V, F, or P). Although not identical, these motifs in both types of CD4 have some distinct similarities. Both of the five-amino acid domains have a cysteine at the center that is found in sequences of all of the species that we examined. The first amino acid in almost all of these motifs is a tryptophan (W), and the fourth and fifth amino acids are almost always aliphatic. The second of the five usually contains an acidic residue but some variation is seen. Regardless of the differences, there is evidence of a possible IL-16-binding motif on both CD4 and CD4–2 molecules. It is interesting to note that, although the lamprey CD4-like molecule has four Ig-like domains, the proximal domain is more similar, including at the putative IL-16-binding site, to teleost two-domain molecules that to conventional CD4 (**Figure 3**).

As previously stated, the lamprey CD4-like molecule does not possess a canonical motif that associates with $p56^{lck}$. Unlike CD4, many cytokine receptors lack a domain for tyrosine kinases. Like two-domain CD4-like molecules, and perhaps the ancestral form of CD4, many cytokine receptors are composed of two immunoglobulin-like extracellular domains and exist as single chains on the surface of cells but, upon encountering an appropriate ligand, form dimers that initiate downstream signaling [65, 66]. The proximal immunoglobulin-like domain is essential to the dimerization of hematopoietic cytokine receptors and involves a motif of four conserved amino acids that resides towards the c-terminal end of the extracellular portion of the molecule and consists of two pairs of conserved

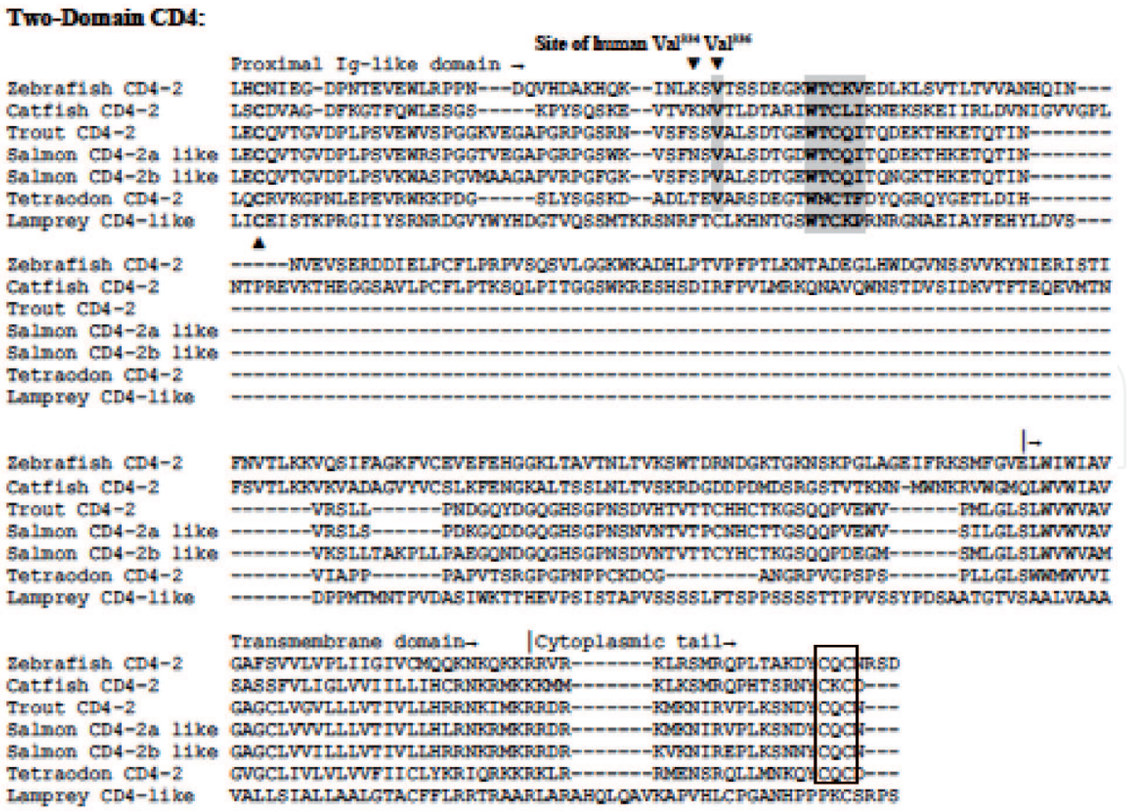

Figure 3.
Multiple alignment (CLUSTALW) of deduced amino acid sequences from proximal domains of CD4–2 from several fish species. Conserved cysteines near the N-terminus of the proximal Ig domain are bolded and marked with an ▲. *A motif similar to IL-16 binding motif of four domain CD4, is shaded, as are conserved leucines that occupy positions similar to the Leu347 required for binding IL-16 on human CD4 [19]. The box surrounds the putative (CxC) binding site for p56lck . Zebrafish CD4–2;*Danio rerio *NCBI accession no. NP_001352990.1, catfish CD4–2:* Ictalurus punctatus *NP_001187156.1, trout:* Oncorhynchus mykiss *XP_021437193.2, Salmon CD4–2a like* Salmo salar *ABZ81914.1, Salmon CD4–2b like* Salmo salar *ABZ81915.1, Tetraodon CD4–2;* Tetraodon nigroviridis *ABU95652.1, lamprey CD4-like* Petromyzon marinus *AAU09669.1.*

amino acids separated by a single, non-conserved residue (WSxWS, [65, 66]). Like these cytokine receptors, CD4 appears to form homodimers by association at the D4 domain [37, 67], the domain that contains the IL-16 binding site. Like cytokine receptors, dimerization of CD4 appears to be a prerequisite to Th activation [67]. Dimerization is also important for effective binding to IL-16, and IL-16 appears to function by bringing CD4 molecules into close proximity [6, 33, 67].

10. Conclusion: probable origins of Gnathostome CD4

Genetic and structural similarities between the D1 and D3 domains and between the D2 and D4 domains give credence to the hypothesis that vertebrate CD4 arose from a precursor with two extracellular immunoglobulin-like domains [36, 61, 64, 68]. In both agnathans and teleost fish, CD4-like molecules with two immunoglobulin domains do not associate with MHC class II molecules but nonetheless appear to be important in immune protection. Lymphocytes with two-domain CD4 molecules, such as those found in teleosts, could very well represent an ancestral $CD4^+$ subset of T cells [61]. The hypothesis that CD4 arose from the duplication of a gene for a protein consisting of two extracellular immunoglobulin domains has been thoroughly discussed and supported [64]. It is quite possible that the physiological importance of these truncated CD4 molecules is that of receptors for IL-16. Regulatory T lymphocytes play a critical role in controlling the immune

response. The gene for IL-16 arose well before the advent of jawed vertebrates and CD4. Although the role of IL-16 in invertebrates has not been clearly elucidated, the ancestral role for CD4 and its evolutionary precursors may be as a receptor for IL-16 that functions to regulate immune function.

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Author details

Gregory D. Maniero
Stonehill College, Easton, MA, USA

*Address all correspondence to: gmaniero@stonehill.edu

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