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PIVOTAL ADVANCE

Pivotal Advance: Avian colony-stimulating factor 1 (*CSF-1*), interleukin-34 (*IL-34*), and *CSF-1* receptor genes and gene products

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ABSTRACT

Macrophages are involved in many aspects of development, host defense, pathology, and homeostasis. Their normal differentiation, proliferation, and survival are controlled by CSF-1 via the activation of the CSF-1R. A recently discovered cytokine, IL-34, was shown to bind the same receptor in humans. Chicken is a widely used model organism in developmental biology, but the factors that control avian myelopoiesis have not been identified previously. The *CSF-1*, *IL-34*, and *CSF1R* genes in chicken and zebra finch were identified from respective genomic/cDNA sequence resources. Comparative analysis of the avian *CSF1R* loci revealed likely orthologs of mammalian macrophage-specific promoters and enhancers, and the *CSF1R* gene is expressed in the developing chick embryo in a pattern consistent with macrophage-specific expression. Chicken CSF-1 and IL-34 were expressed in HEK293 cells and shown to elicit macrophage growth from chicken BM cells in culture. Comparative sequence and co-evolution analysis across all vertebrates suggests that the two ligands interact with distinct regions of the CSF1R. These studies demonstrate that there are two separate ligands for a functional CSF1R across all vertebrates. ***J. Leukoc. Biol.*** **87: 000–000; 2010.**

Introduction

Macrophages are derived from progenitor cells in the BM, which have differentiated into blood monocytes and then entered the tissues to become resident [1], where they constitute the first line of defense against pathogens, maintain homeostasis, and have trophic functions ranging from bone morphogenesis to neuronal patterning in sexual development and from angiogenesis to adipogenesis [2]. CSF-1 is required for normal differentiation, proliferation, and survival of macrophage-lineage cells [3–5]. Mice and rats bearing mutations in the *CSF-1* gene (i.e., *op/op* mice and *tl/tl* rats) show a mononuclear phagocyte deficiency, and their important developmental abnormalities, such as reduced somatic growth, perinatal mortality, osteopetrosis, and neurological and reproductive defects, highlight many of the macrophage-trophic roles mentioned above [6–8].

Although CSF-1 exists in a number of isoforms, most biochemical studies have focused on the smallest fragment with biological activity, i.e., the 154 N-terminal aa, common to all isoforms. In a full-length CSF-1 molecule, this receptor-binding region is preceded by a 32-aa signal peptide and followed by a variable spacer region (encoded by Exon 6, an alternatively spliced exon), a 24-aa transmembrane region, and a 35-aa cytoplasmic tail. The tertiary structure of biologically active CSF-1 forms a short-chain four-helical bundle (A, B, C, and D) with small regions of β-sheet (1 and 2). The helices are paired into A–C and B–D by intrachain disulfide bonds, and one interchain disulfide bond generates a mature homodimer with a twofold rotation axis [9]. The crystal structure of mouse CSF-1, bound to CSF1R, was solved recently to a resolution of 2.4 Å, showing that the CSF-1 N-terminal segment (residues 6–15), helix B (residues 55–66), and helix C (residues 79–85) are implicated in receptor binding (PDB Code 3ejj) [10].

All CSF-1 effects are mediated through binding to the CSF-1R, a glycoprotein of 165 kDa that is encoded by the *c-fms* proto-oncogene [11]. CSF1R is a member of the type III protein tyrosine kinase family, along with PDGFRA, PDGFRB, and c-kit, and is therefore composed of a charac-

Abbreviations: 3D=three dimensional, AML1=acute myeloid leukemia 1, BMM=bone marrow-derived macrophage(s), c=circulating, CAPS=co-evolution analysis using protein sequence, CDS=coding sequence, DEPC=diethylpyrocarbonate, d_N/d_S=nonsynonymous/synonymous substitution, Ets=E26, FIRE=Fms intronic regulatory element, GH=growth hormone, GHR=GH receptor, HEK=human embryo kidney, HH=Hamburger and Hamilton, HI=heat-inactivated, ISH=in situ hybridization, ORF=open-reading frame, PDB=Protein Data Bank, PDGFRA/B=platelet-derived growth factor receptor A/B, SCF=stem cell factor

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teristic extracellular region of five Ig-like domains (D1–D5), a single transmembrane helix, and an intracellular tyrosine kinase domain [12]. CSF-1 associates tightly with the receptor (K_D=0.4 nM at 37°C) in a 2:2 stoichiometry [13]. This binding involves the cleft between Domains 2 and 3; the site is formed from the CD loop (Residues 141–151) and the EF loop (Residues 168–173) of Domain 2, as well as the BC and DE loops (Residues 231–232 and 250–257, respectively) of Domain 3 [10].

The expression of CSF1R on the cell surface is amongst the earliest events in macrophage-lineage commitment and is restricted mostly to these cells, not only throughout embryonic development but also in adults [14] Like other myeloid promoters, the proximal *CSF1R* promoter lacks the classic TATA box and GC-rich sequences but contains recognition sites for AML1 transcription factors and for transcription factors of the C/EBP and Ets families, including the myeloid-restricted transcription factor PU.1 [4, 15, 16]. Expression of CSF1R is also controlled by *FIRE*, a highly conserved enhancer element in the first intron [17, 18].

Most of the phenotypic defects seen in the *op/op* mice, including reproductive defects and perturbations in organ development, are even more severe in the *CSF1R* knockout mice [11]. One explanation involved the availability of maternal-derived CSF-1 [19]; another was that CSF1R might respond in a CSF-1-independent manner in some circumstances [11]. These observations can now be explained by the recent discovery of a second ligand for the human CSF1R, designated IL-34, which like CSF-1, was shown to regulate human monocyte viability in vitro. IL-34 was purified as a homodimer composed of 241 aa monomers and shown to be expressed at detectable levels in many human tissues, including heart, brain, spleen, lung, liver, kidney, and thymus [20].

The chicken has been used widely in studies of early embryonic myelopoiesis [14, 21], but compared with our knowledge of the mammalian mononuclear phagocyte system, our knowledge of avian systems is rather limited. There are only two characterized colony-stimulating factors in chickens. Chicken *GM-CSF* (*CSF2*) has been cloned and shown to drive the proliferation of chicken BM cells [22]. The other described chicken CSF, myelomonocytic growth factor, has been shown recently to be the chicken ortholog of *G-CSF* (*CSF3*) [23]. Apparent orthologs of the *GM-CSFR* α-chain and β-chain, shared with the *IL-3R*, are annotated in the chicken genome (www.ensembl.org). So far, no function for the CSF1R in birds has been demonstrated. Factors in post-endotoxin chicken lung were able to elicit macrophage growth from chicken marrow and were reactive in a radioimmunoassay against mouse CSF-1 [24]. However, a gene encoding an avian ortholog of *CSF-1* was believed to be absent from avian genomes [25], and *IL-34* had also not been recognized.

CSF-1 and IL-34 in humans were reported to have little obvious homology, and in mammals, at least, CSF-1 has evolved rather rapidly. The existence of two ligands for a single receptor is difficult to maintain across evolution if they are potentially redundant and if each can evolve independently of the receptor. Herein, we show that *CSF-1* and *IL-34* are present in birds, the *CSF1R* gene is expressed in macrophages, and both ligands can promote macrophage proliferation from avian BM cells. Structural modeling reveals that both factors are, in fact, four-helix bundles. The known contact residues in CSF-1 are divergent across species, and within a species, they are not conserved between CSF-1 and IL-34. Analysis of the co-evolution of the two growth factors with the receptor suggests that they must bind in different locations, which would allow each to evolve independently. The chick provides a model in which to investigate the biological functions of these two factors in development of the mononuclear phagocyte system.

MATERIALS AND METHODS

Bioinformatics analysis

Nucleotide and peptide sequences were identified using the databases at National Center for Biotechnology Information (Bethesda, MD, USA) and the genome resources from the University of Santa Cruz (Santa Cruz, CA, USA) and Ensembl: www.ncbi.nlm.nih.gov/index.html, http://genome.ucsc.edu, and www.ensembl.org/index.html. The peptide sequence of the zebra finch *CSF1R* gene was predicted using the GeneWise program (www.ebi.ac.uk/Tools/Wise2/index.html), and the chicken *IL-34* expressed sequence tag was analyzed using ESTScan (www.ch.embnet.org/software/ESTScan2.html).

Cloning of chicken and zebra finch cDNA genes

RNA samples from chicken stage 20 HH embryo and zebra finch brain were extracted using TRIzol reagent, as described by the manufacturer (Invitrogen, Paisley, UK). Specific cDNAs were cloned via RT-PCR using Superscript III RT and the TOPO TA cloning kit for sequencing (Invitrogen). 5′ RACE of chicken and zebra finch CSF-1 and zebra finch IL-34 was carried out using the FirstChoice RNA ligase-mediated-RACE kit (Ambion, Warrington, UK). PCR products were cloned using the TOPO TA cloning kit for sequencing (Invitrogen). The 3′ end of chicken CSF-1 was cloned using a modified 3′ RACE technique, 3′ RACE LaNe [26].

Genetic mapping of chicken CSF-1

A 437-bp genomic fragment of the chicken CSF-1 gene was amplified using the primers ckcsfex4-for1: GCGACTCTGTCTGCTACGTG and ckcsfex5-rev1: CGAAGGTCTCCTTGTTCTGC. Sequencing of the parental DNA from the East Lansing reference population [27] identified a single nucleotide polymorphism in Exon 4 (G/A in Red Jungle Fowl male; A/A in White Leghorn female), and sequencing of 52 backcross progeny DNAs confirmed that the CSF-1 gene mapped to Chr26. Linkage analysis was carried out using the Map Manager program [28].

Phylogenetic analyses

Amino-acid sequences of annotated CSF-1, IL-34, and CSF1R genes from various taxa were aligned using the ClustalW software [29] (Gonnet protein weight matrix, no end gaps inactivated, default parameters). The secondary structures shown in the structure-based alignments were predicted using PSIPRED [30], and the alignments were performed by Domain Fishing [31].

Whole-mount ISH

Fertilized White Leghorn eggs were collected weekly and incubated at 38°C between 3 and 6 days of development. Embryos were dissected into cold DEPC-PBS, staged as per [32], fixed immediately in 4% paraformaldehyde/DEPC-PBS overnight, dehydrated into 100% methanol through graded methanol/PBS steps, and stored at –20°C. Whole-mount

ISH on embryos was carried out as per [33]. The CSF1R probe was made using the ARK-Genomics (The Roslin Institute, UK) clone chEST654k20 as a template.

Expression cloning of chicken CSF-1 and IL-34 peptides

HEK293T cells (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM (Sigma Chemical Co., St. Louis, MO, USA), supplemented with 10% HI-FCS, 2 mM L-glutamine, 0.1 mM nonessential aa, and antibiotics (100 ug/ml penicillin and 100 ug/ml streptomycin). One day before transfection, HEK293T cells were plated and transfected with Lipofectamine 2000, as per product instructions. Cells were then incubated at 37°C in a CO_2 incubator for 48 h prior to harvesting the supernatant and lysing the cells in 2% SDS-10 mM Tris buffer. The cell extracts and supernatants were then mixed with Laemmli buffer, with or without DTT (5 mM final) or B-ME (5% final), run on a 4–12% gradient SDS-PAGE gel, and transferred on polyvinylidene difluoride membrane, as per Bio-Rad apparatus instructions (Bio-Rad, Hercules, CA, USA). The membrane was blotted using a mouse anti-v5 tag antibody (AbD Serotec, Raleigh, NC, USA) and an anti-mouse IgG HRP conjugated (Cell Signaling Technology, Beverly, MA, USA).

BM differentiation

Chicken BM cells were obtained by flushing the marrow from two femurs and two tibias with PBS using a syringe and a blunt needle. For each condition, one of 250 total cells was pelleted and resuspended in 4 ml complete RPMI (supplemented with 10% HI-FCS, 2 mM L-glutamine, 100 ug/ml penicillin, and 100 ug/ml streptomycin) containing 20% supernatant from empty pEF6-, pEF6-cCSF-1-, or pEF6-cIL-34-transfected HEK293T. Cells were plated in 60 mm bacteriological plates and incubated at 37°C in a CO_2 incubator for 12 days.

3D structure predictions of CS1, IL-34, and CSF1R proteins

3D models in PDB format were generated with 3D-Jigsaw (http://bmm.cancerresearchuk.org/3djigsaw/) using structure-based alignments (performed by Domain Fishing) [34]. The PDB files obtained were viewed in FirstGlance in Jmol (http://firstglance.jmol.org), and the models were rendered by PyMol using Polyview-3D (http://polyview.cchmc.org/polyview3d.html) [35].

Intra- and intermolecular co-evolution analysis

To identify co-evolutionary patterns, we used the parametric method based on correlated evolutionary patterns among amino-acid sites [36]. To perform the analysis, we used the software implementing this method CAPS (Version 1.0) [37], which has proven to be successful in yielding meaningful results in several case studies, including those aimed at identifying co-evolution of membrane proteins [38], HIV gp120 and gp41 proteins [39], as well as the Hsp70-Hop-Hsp90 system [39]. To estimate the probabilities and significance of the correlated evolutionary patterns among amino-acid sites, we used a large number of random samplings (1 million and 10 million random samples) and a small α value (0.001) to minimize a false-positive rate (type I error). CAPS also implements the step-down permutational procedure, as described previously [39], to correct for multiple testing. The scores for the amino-acid substitutions were obtained using the appropriate blocks substitution matrix (BLOSUM80) [40], depending on the similarity of our protein sequences. All amino-acid sites reported in the co-evolutionary analyses present the positions in the protein from the reference sequence (human).

Visualization of co-evolutionary networks

We used the software Cytoscape (Version 2.6.1) [41] to visualize the co-evolutionary networks identified by CAPS. Cytoscape was designed originally to visualize bimolecular interaction networks. This tool, however, can be used to visualize any data that describes interactions between objects. CAPS can produce four files containing information of co-evolutionary networks and compensatory mutations. We used this program to generate the networks of correlation between co-evolving amino acids and used the correlation coefficients generated in CAPS to determine the coloring patterns of the linking lines between nodes (amino-acid residues).

RESULTS

Identification of avian *CSF-1* genes

There was no annotated *CSF-1* gene previously in the chicken genome, but the region containing the mouse *CSF-1* gene displayed conserved synteny with the chicken, suggesting that there was a gap in the chicken genome assembly. Within the zebra finch genomic sequence, it was possible to identify a partial *CSF-1* ortholog (Chr26: 24,187–27,419, July 2008 assembly), as described in Materials and Methods. A complete ORF was obtained by 5′ RACE to determine the full CDS. This, in turn, led to the identification of a chicken trace sequence (shq36e03.b1), which was similarly extended by 5′ and 3′ RACE to give the full CDS. *CSF-1*-containing bacterial artificial chromosomes were also identified, end-sequenced, and mapped to Chr26 genomic sequences. A 437-bp PCR fragment specific for the chicken *CSF-1* gene was amplified from genomic DNA from the East Lansing backcross panel and used to map this gene genetically to Chr26. The newly identified avian *CSF-1* genes each contain eight exons. The zebra finch gene (GQ249405) encodes a protein of 489 aa, and the chicken gene (GQ249403) encodes a protein of 490 aa. We have also identified a second chicken transcript that comprises 270 aa (GQ249404). The shorter transcript is missing a substantial part of Exon 6. In mammals, this exon encodes a large domain that contains a proteolytic cleavage site, which permits the release of CSF-1 from a membrane-anchored precursor. The shorter transcript might therefore encode a chicken ortholog of the membrane-anchored cell surface form of CSF-1, which cannot be cleaved. The Exon 6 of chicken CSF-1 also contains the unique glycosaminoglycan (chondroitin sulfate) addition site (SGXG/A) found in the mammalian genes. Although functional confirmation awaits, the sequence data indicate that the basic biology of CSF-1, involving secreted and membrane-anchored forms with variable post-translational modification and distinct functions and different glycoforms [42–44], is likely to be conserved in the chicken.

Conserved structure of CSF-1 protein

To assess whether the avian CSF-1 sequences identified are likely functional orthologs of mammalian CSF-1, a multiple alignment of deduced amino-acid sequences across many species was performed using the ClustalW software [29] (Supplemental Material and Supplemental Table 1). The six cysteine residues responsible for the three intramolecular disulfide bonds [9] are conserved in birds, but the cysteine forming the interchain disulfide bond that is located at the dimer interface and conserved through all nonavian species, including zebrafish and goldfish [45], is not conserved in birds. The alignment of the cysteines and predicted helices

highlights the contact residues for CSF-1 bound to CSF1R, deduced from the co-crystal in mouse [10], some of which are clearly divergent in birds. In particular, the D91 and D94, Q113, E114, and N117 (position numbers referring to the mouse sequence in Supplemental Table 1) are not conserved or semi-conserved substitutions. Immediately downstream of these binding sites, the bird sequences have additional amino acids that are not present in the mammalian sequences. The avian CSF-1 proteins are clearly very divergent from mammalian proteins; it is not clear that chicken CSF-1 was the likely component that cross-reacted with a radioimmunoassay of mouse CSF-1 [24]. This alignment also highlights the substitution of R111 in mouse and Q in human, which could explain why the human ligand is active on mouse cells but not vice versa [4].

To refine the avian CSF-1 structure predictions, 3D models in PDB format were generated as outlined in Materials and Methods. The avian CSF-1 molecules are predicted to have the same four-helix bundle structure as the well-described mammalian CSF-1 [9]. In **Figure 1A**, the chicken CSF-1 model is compared with the published mouse structure [10]. Although the overall topology is conserved from mammals through birds, all of the differences found in the sequence alignment translate into structural changes. Hence, the predicted CSF1R-binding site 1 of chicken CSF-1 comprises different charges from the mouse-binding site 1, and these nonconserved amino-acid substitutions are positioned precisely to contact with the receptor (red arrows). Moreover, the extra residues found in the chicken sequence are predicted to create a protuberance, making the positively charged R122 stick out between binding sites 1 and 2 (black arrow). As mentioned above, the cysteine forming the interchain disulfide bond in mammals (labeled Cys on the mouse structure) is absent from the chicken CSF-1. The absence of a disulfide link is also the case for the closely related SCF in mammals, as well as in birds [46]. SCF proteins are able to form stable dimers and induce receptor dimerization because of the large contact surface area between the interacting monomers [47]. If one assumes the same topology of dimer interaction in avian CSF-1 as in mammalian CSF-1 and SCF, the predicted contact surface between the chicken CSF-1 monomers is similar to that of chicken SCF. They both contain exposed hydrophobic residues, as shown in Figure 1B, where the amino acids present on the proposed dimer interface were rendered as spacefill. Hence, we predict that avian CSF-1 will form noncovalent dimmers, despite the absence of any potential interchain disulfide bridge.

Identification of avian *IL-34* genes

There is an obvious candidate chicken ortholog of human *IL-34* in the chicken genome, and we have identified a chicken cDNA within the ARK-Genomics (The Roslin Institute) collection, which maps to Chr11 and contains the full-length *IL-34* CDS (GenBank Accession No. BX931154). This chicken sequence permitted the identification of the orthologous zebra finch gene within the genome sequence (Chr11: 5,575,502–5,577,560; July 2008 assembly; GQ249406). The avian genes each contain six exons, the chicken gene encoding a protein of 178 aa and the zebra finch gene encoding 180 aa.

Conserved structure of IL-34 protein

At the amino-acid sequence level, IL-34 is considerably more conserved amongst avian and mammalian species than CSF-1 (Supplemental Material and Supplemental Table 2). The paper describing human *IL-34* suggested that it was structurally novel [20]. Mammalian and avian IL-34 and CSF-1 peptide sequences, however, can be aligned with each other, showing weak primary homology but similar predicted secondary structure, strongly suggesting that IL-34 is also a four-helix bundle (data not shown). To refine this view, a 3D model of the chicken IL-34 was generated as outlined in Materials and Methods and is shown in **Figure 2A**. For structural comparison, a model for the chicken SCF was created following the same procedure, and the chicken CSF-1 model shown in Figure 1 was also included in Figure 2A. This structural analysis of the derived protein sequence of IL-34 is consistent with a four-helix bundle structure. However, it predicts a molecule that lacks all seven strategically positioned cysteines of CSF-1. IL-34 contains some generally conserved cysteine residues, but

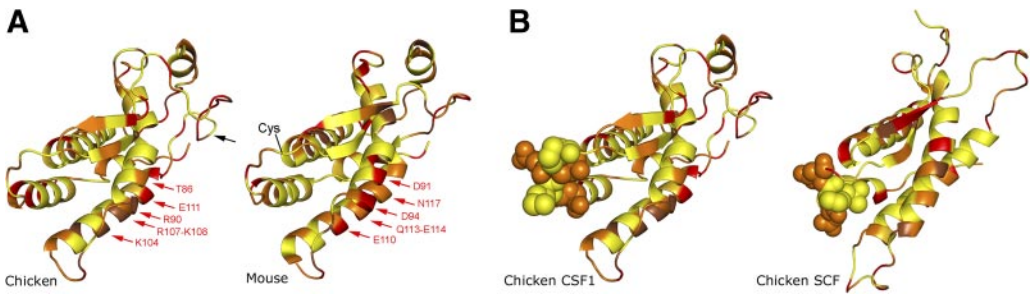

Figure 1. Structure-based sequence analysis of chicken CSF-1. (A) Ribbons representation of the chicken (left) and mouse (right) CSF-1 structures. The file for the chicken CSF-1 was generated using 3D-Jigsaw with the mouse CSF-1 structure as template (PDB Code 3ejj). The figures were rendered by PyMol in Polyview-3D. Residues are colored according to hydrophilicity, where yellow is used for hydrophobic residues (A, C, F, G, I, L, M, P, V), dark yellow for amphipathic residues (H, W, Y), orange for polar residues (N, Q, S, T), red for residues charged negatively (D, E), and brown for residues charged positively (R, K). The cysteine forming the interchain disulfide bond is labeled on the mouse structure. The loop created by the presence of additional amino acids in the chicken sequence is marked by a black arrow. The red arrows show nonconserved substitutions within the CSF1R-binding site 1, as described in the mouse [10]. (B) Dimer interface of chicken CSF-1 (left) and SCF (right). Residues are colored as in A. Amino acids present at the homodimer interface are highlighted by rendering their atoms as spheres.

these are not positioned and matched together to form intrachain disulfide bonds. As noted above, human IL-34 was purified as a homodimer. The cysteine required for the formation of an interchain disulfide bridge is not found on the predicted dimer interface (assuming the same dimer orientation as CSF-1). However, that region does contain exposed hydrophobic residues (Fig. 2B). Red arrows point out the corresponding amino acids that constitute the CSF1R-binding site 1 on CSF-1 (Fig. 2A). These residues are totally different from those in the corresponding positions within IL-34, immediately suggesting an alternate binding mechanism from that of CSF-1.

Identification of the zebra finch *CSF1R* gene

In the chicken genome on Chr13, there is an annotated *CSF1R* gene (Ensembl ID: ENSGALG00000005725) occupying the same position as in mammals, immediately 3′ of the closely related *PDGFRB* gene (Ensembl ID: ENSGALG00000021313). The availability of the chicken sequence allowed us to identify the orthologous sequence in the zebra finch genome (Chr13: 6,954,381–6,972,446; July 2008 assembly; GQ249407). This gene contains 21 exons and codes for a protein of 967 aa, which is the same as the chicken *CSF1R*.

Evolutionary conservation of CSF1R protein

A multiple alignment of amino-acid sequences was performed using the ClustalW software to examine the homology between the two avian CSF1Rs and mammalian orthologs (Supplemental Material and Supplemental Table 3). As expected, the intracellular tyrosine kinase domain (Residues 540–977) of the receptor is extremely conserved in all species, including birds. Amongst the residues that are not conserved, however, is cysteine 665 (indicated by an arrow in Supplemental Table 3), which is substituted in birds by an arginine. This substitution may underlie our finding that chicken CSF-1-induced macrophage growth is not blocked by a kinase inhibitor, GW2580 (V. Garceau, unpublished results), which inhibits mammalian CSF1R activity [48]. The structure of the mammalian kinase domain bound to inhibitor is available (PDB 3BEA), and this is the only contact amino acid that is not conserved in birds.

Most of the residues comprising the CSF-1-binding sites 1 and 2 (labeled, respectively, with "+" and "o" symbols in Supplemental Table 3), deduced from the mouse CSF1R/CSF-1 co-crystal, are actually not well-conserved between mammalian or avian species. Some of the amino-acid substitutions observed between the two birds are illustrated in **Figure 3A**, where 3D models of the CSF-1-binding site 1 on the D2 domain of the zebra finch (left) and the chicken (right) CSF1R are presented. As with the ligands, the models were created using 3D-Jigsaw and Polyview-3D. A more general view of the D1–D3 domains of these same receptors with a superimposed structure of their respective CSF-1 molecule is presented in Figure 3B. The receptors are shown from the same angle as in Figure 3A, and the predicted chicken CSF-1 structure is the same model as in Figure 1A, rendered as surface instead of cartoon, with the dimer interface labeled for orientation. The predicted zebra finch CSF-1 structure was produced using the same settings, and the superimposition angle is based on the co-structure of the mouse CSF-1:CSF1R complex [10]. In Figure 3B, the amino-acid substitutions in the zebra finch (left) and chicken (right) receptors are put in context with those present in their respective CSF-1 ligand. This high level of divergence is not surprising, as zebra finches and chickens are not part of the same taxonomic order. As with primate and rodent CSF-1, we predict that the zebra finch CSF-1 will not activate the chicken receptor, or vice versa, as a result of the important changes in charge density in the binding sites.

Positive selection of specific amino acids in CSF-1, IL-34, and CSF1R

Genes involved in innate immunity are under strong evolutionary selection. For example, even where there is clear orthology between mouse and human genes, the average d_N/d_S ratio is generally three- to fourfold higher in immune-related genes [49]. A comparison of such genes across species is provided at ImmTree (http://bioinf.uta.fi/ImmTree) [50].

To evaluate how fast each of the genes of interest in this study is evolving, we first estimated $\omega = d_N/d_S$ ratios between pairs of species using CODEML within the Phyloge-

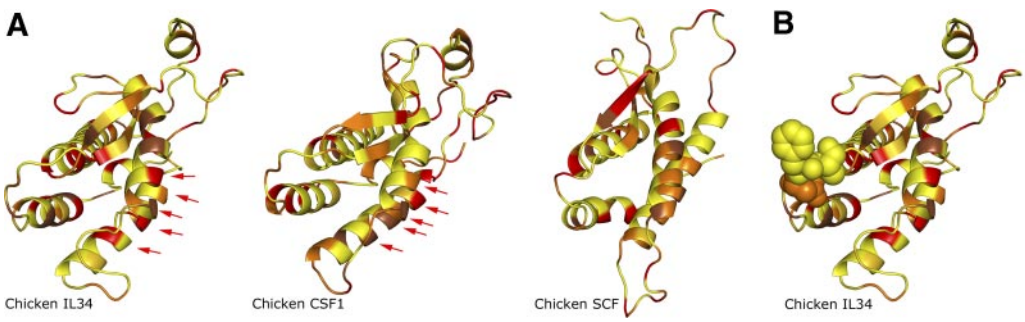

Figure 2. Structure-based sequence analysis of the chicken IL-34. (A) Ribbons representation of the chicken IL-34 (left). Structures of the chicken CSF-1 (center) and chicken SCF (right) are shown for comparison. The PDB file for the chicken IL-34 was generated using 3D-Jigsaw with the mouse CSF-1 structure as template (PDB Code 3ejj). The PDB file for the chicken SCF was generated using 3D-Jigsaw with the human SCF structure as template (PDB Code 1exz). The figures were rendered by PyMol in Polyview-3D. Residues are colored as in Figure 1A. The red arrows highlight the corresponding residues composing the receptor-binding site 1 in CSF-1. (B) Dimer interface of chicken IL-34. Residues are colored as in A. Amino acids present at the homodimer interface are highlighted by rendering their atoms as spheres.

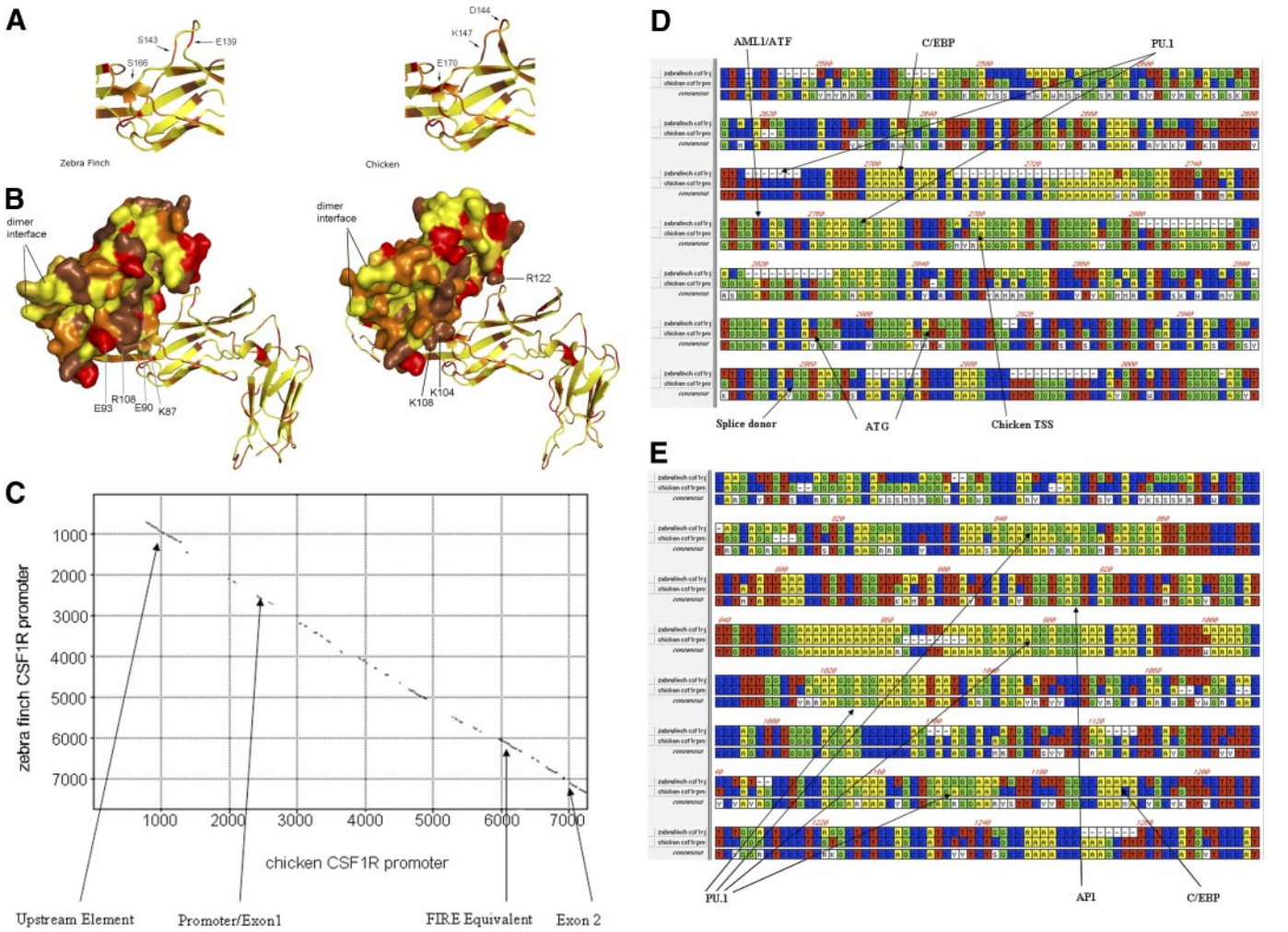

Figure 3. Characterization of the avian CSF1R. (A) Ribbons representation of CSF-1-binding site 1 of the zebra finch (left) and chicken (right) CSF1R. The PDB files for the chicken and zebra finch CSF1R were created with 3D-Jigsaw using the mouse CSF1R structure as template (PDB Code 3ejj). The figures were rendered by PyMol in Polyview-3D. Residues are colored as in Figure 1A. Arrows point at some of the nonconserved amino-acid substitutions. (B) Superimposition of the zebra finch (left) and chicken (right) D1–D3 domains of CSF1R with their respective CSF-1 ligand. The two CSF1R structures are viewed from the same angle as in A. The chicken CSF-1 structure from Figure 1A is here rendered as surface in Polyview-3D. The PDB file for the zebra finch CSF-1 structure was created with 3D-Jigsaw using the mouse CSF-1 as template (PDB Code 3ejj) and rendered with Polyview-3D. Residues are colored as in Figure 1A. (C) Pustell DNA matrix alignment of the 5′ ends of the avian CSF1R genes. The two genes align up to 2.5 kb upstream and through the first intron. (D) Sequence alignment of the promoter-Exon 1 region of the zebra finch and chicken CSF1R gene. Alignment was performed using MacVector. Binding sites for PU.1, C/EBP, and AML1 are identified. ATF, Activating transcription factor; TSS, transcription start site. (E) Alignment of the highly conserved segment in the intron of the zebra finch and chicken CSF1R gene. Alignment was performed as in D. Binding sites for PU.1, C/EBP, and AML1 are identified. (F) Localization of *CSF1R* mRNA in chick embryo at 20 HH by whole-mount *ISH*. The figure shows the antisense (left) and the sense control ISH (right).

netic Analysis by Maximum Likelihood package [51]. For zebra finch- and chicken-coding sequences, the ratio for CSF1R is 0.27, CSF-1 is 0.25, and IL-34 is 0.09. In general, when zebra finch and chicken genes are compared, the average ω is 0.1 (D. W. Burt, unpublished results). Thus, CSF1R and CSF-1 have the higher values typical of rapidly evolving genes involved in immunity, whereas IL-34 has a lower value, which would suggest that it is under evolutionary constraint to remain relatively unchanged. For comparison, ω ratios for mammals from the Ensembl database are: CSF-1 is 0.31–0.82, IL-34 is 0.14–0.66, and CSF1R is 0.13–0.33 against a genome-wide average of 0.05.

Transcriptional regulation of avian *CSF1R*

The mammalian CSF1R loci contain a conserved macrophage-specific promoter region and a remarkably conserved enhancer (FIRE) in the first intron that is required for macrophage-specific expression of a transgene [18]. These elements are not evidently conserved in birds. However, the factors required for macrophage gene expression, such as PU.1, have clear chicken orthologs. To assess the likelihood of CSF1R being a macrophage regulator in birds, we first assessed the transcriptional regulation of the chicken and zebra finch genes. Passeriformes and galliformes are separated by approximately 100 million years of evolution, about the same as rodents and humans (www.timetree.org). Because of this distance, evolutionary conserved, noncoding regions provide strong indications of the location of functional promoters and enhancers; in mammals, the FIRE is more conserved than any of the exons [17]. The intron-exon structure of avian *CSF1R* is the same as in mammals (www.ensembl.org), and the ATG start codon is located in the first exon. Like the mammalian *CSF1R* promoters, the two avian *CSF1R* proximal promoters are not highly conserved, but both are purine-rich and TATA-less. In both bird species, there are two highly conserved regions: upstream and downstream of the promoter. This is shown in the Pustell DNA matrix alignment of Figure 3C. The downstream element is in the same relative location within the first intron as the mammalian FIRE [17]. The candidate avian FIRE sequence cannot be aligned with the mammalian FIRE but contains the same basic elements. These regions contain multiple repeats of consensus sequences in common with mammalian *CSF1R* and FIRE, including candidate-binding sites for PU.1 and other Ets factors, AP1, C/EBP, specificity protein 1, and AML1 (Fig. 3, D and E). Each of these transcription factors is expressed in hematopoietic cells in chickens [52, 53]. These findings suggest that avian CSF1R is controlled in basically the same manner as in mammals, despite the reassortment of the cis-acting individual elements.

Expression pattern of *CSF1R* in chick embryo

The analysis of conserved regions identifies candidate enhancers of the chicken *CSF1R* locus and suggests that the gene is likely to be expressed specifically in macrophages. To confirm that prediction, we carried out whole-mount ISH of chicken embryos at 20 HH or 3.5 days of development, the wing-bud stage (Fig. 3F). This stage corresponds to 11.5 days post-coitum in the mouse embryo. In the mouse, *CSF1R* and many other macrophage-expressed genes are expressed in individual cells throughout the embryo, associated particularly with regions of high cell death [14]. The distribution of macrophage-restricted mRNAs is similar in *Xenopus* [54]. The chicken *csf1r* mRNA was also expressed in single cells that form a speckled pattern all over the embryo (left panel). No other cell type is known to be distributed in this manner, and the pattern confirms simultaneously that the mRNA is not expressed in any nonhematopoietic cell type or tissue during development.

Activation of the chicken CSF1R by CSF-1 and IL-34

The expression of CSF1R on the cell surface is amongst the earliest events in macrophage-lineage commitment [55], and CSF-1 is commonly used to grow pure populations of macrophages from mouse BM [4]. To confirm that the avian CSF1R ligands are both active, they were therefore tested for differentiation of chicken BM cells. The chicken CSF-1 (first six exons) and IL-34-predicted ORFs were cloned in the pEF6 vector, providing them with a C-terminal tag for detection, and transfected into HEK293T cells. The expressed proteins within the cell and supernatant were detected by Western blotting (**Fig. 4A**). In the case of CSF-1, two apparent molecular entities were seen in nonreduced and reduced supernatant, and the lowest apparent MW (ca., 60 kDa) is mostly present in the cells, suggestive that the secreted protein might be processed and glycosylated before its secretion, possibly even as a proteoglycan. In the case of IL-34, the protein was detected in the cells and supernatant as a doublet, and the higher apparent MW was more abundant than the lower MW, suggesting that IL-34 might also be glycosylated in this system, as is the human IL-34 protein expressed in HEK cells [20]. The amount of chicken IL-34 detected in the supernatant was considerably less than seen in the cell lysates. This might be a result of some proteolytic cleavage by trypsin-like proteases in the medium, as many recognition sites are found at the C-terminal of IL-34, just upstream of the v5 tag. The supernatant from the transfected HEK293T cells or cells transfected with the vector

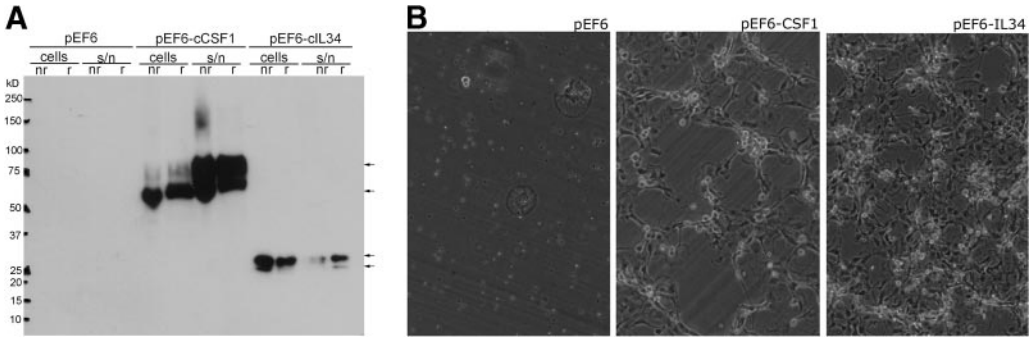

Figure 4. Expression and activity of the chicken CSF-1 and IL-34. (A) Expression and secretion of chicken CSF-1 and IL-34 by HEK293T transfected with pEF6-cCSF-1, pEF6-cIL-34, or empty pEF6 vector. The cells were transfected using Lipofectamine and incubated for 72 h. The cell lysates and supernatants were run on SDS-PAGE gel in nonreducing and reducing conditions and probed with an anti-V5 tag antibody. The arrows show the double bands for the chicken CSF-1 (ca., 60 kDa) and IL-34 (ca., 25 kDa), in which the upper bands are the glycosylated forms of these proteins, whereas the lower bands constitute the nonglycosylated forms. s/n, Supernatant; nr, not reduced; r, reduced. (B) Chicken BMM at Day 10 of differentiation using 20% supernatant from pEF6 (left)-, pEF6-cCSF-1 (center)-, and pEF6-cIL-34 (right)-transfected HEK293T.

only was added to BM cells obtained by flushing the marrow from a chicken femur, and the cells were incubated for 10 days. At approximately Day 3, cells growing in the presence of chicken CSF-1 or IL-34 became adherent, and by Day 12, the dishes were confluent. No BM cells survived in the control dish containing supernatant from empty pEF6-transfected HEK (Fig. 4B). Hence, CSF-1 and IL-34 offer the possibility of growing macrophages from chicken marrow for functional studies; BMM have been a mainstay of macrophage functional studies in the mouse [4]. To confirm that these activities are mediated through the CSF1R, we have transfected chicken CSF1R stably into the factor-dependent Ba/F3 cell line used previously in studies of the mouse and human CSF1R [48] and shown that the resulting cells proliferate in the chicken CSF-1- or IL-34-containing HEK293 supernatant (data not shown). Together, the data indicate that the function of CSF1R and its two ligands is conserved in birds and mammals.

Co-evolution of CSF1R, CSF-1, and IL-34 proteins

The extracellular domain of CSF1R is quite divergent among species, as is one of its ligands, CSF-1. Even between mice and rats, IL-34 is considerably better-conserved and yet, activates the same receptor as CSF-1. The divergence of CSF-1/CSF1R could be related to the fact that CSF-1 is massively inducible in the circulation in response to innate-immune stimuli such as LPS, and the CSF1R extracellular domain is cleaved from the cell surface in cells responding to a range of TLR agonists through the actions of a disintegrin and metalloprotease domain 14/TNF-α-converting enzyme [56, 57]. So, one might argue that CSF-1 is required for effective innate-immune responses and is therefore under intense immune selection. Indeed, EBV encodes a soluble CSF1R antagonist, BARF1 [58]. This raises the interesting evolutionary question about exactly how two ligands could evolve with a single receptor. In theory, and provided the overall structure is conserved, alterations in contact residues in the ligand should be compensated by alterations in the receptor to preserve binding affinity, and within a sufficiently large sample, there should be a correlation matrix between amino acids on the partners. Incorporating the alignments presented in Supplemental Tables 1–3 and published PDB structures for CSF-1/CSF1R, it was possible to distinguish functional co-evolution from phylogenetic or random co-variation to calculate a correlation coefficient [36]. The CAPS software was used to identify amino-acid sites having a strong correlation coefficient [37], and the networks of these co-evolving residues are shown in **Figure 5A**. The strength of the correlation coefficient for specific site pairs is indicated by the color of the line connecting them, and the amino-acid position numbering follows the human sequences as reference. Unexpectedly, the only significant correlations were found between CSF1R and the more conserved of the two ligands, IL-34. Moreover, no sign of intra-protein co-evolution was found in any of the three proteins.

IL-34-binding mode of CSF1R

None of the co-evolving amino-acid positions identified by CAPS in CSF1R is located within the CSF-1-binding sites in the D2 and D3 domains [10]. Instead, they are concentrated at the junction between D3 and D4. In a similar manner, the co-evolving residues in IL-34 are located outside of the corresponding CSF1R-binding site of CSF-1. All of the co-evolving residues appearing in the networks of Figure 5A were mapped on the chicken CSF1R and IL-34 structures (Fig. 5B). The chicken CSF1R D1–D5 structure was generated as a chimera of two PDB files generated by 3D-Jigsaw for the chicken receptor: D1–D3 using the mouse CSF1R structure as template (PDB Code 3ejj) and D1–D5 using the human KIT structure as template (PDB Code 2e9w). The two models were then superimposed to create a model of the chicken CSF1R D1–D5 structure. The chicken IL-34 model is the same one as in Figure 2, viewed from a slightly different angle. The corresponding co-evolving residues in chicken were deduced from the alignment in Table 2 and then highlighted in blue using Polyview-3D. The co-evolution of specific sites on CSF1R and IL-34 can be interpreted as a possible binding mode between this uncharacterized new ligand and its receptor. Hence, we can speculate that the IL-34:CSF1R-binding site interface consists of the CD loop of IL-34 and the region around the D3–D4 junction of CSF1R.

DISCUSSION

The structural basis of the interaction among CSF-1, IL-34, and CSF1R proteins presents a scientifically interesting phenomenon from an evolutionary point of view. Current genomic efforts now provide peptide predictions from more than 15 species for ligands and their receptor. From these sequences, it is apparent that the interaction is conserved as far back as our fish ancestors, 450 million years ago. By aligning the sequences and examining the tolerance of different residues at significant positions, it is possible to identify particular amino acids in the receptor that vary in conjunction with a given residue for CSF-1 or IL-34 ligands. The ability of CSF-1 from one species to induce growth and survival of macrophages (or cells transfected with the receptor) from another species adds weight to predictions, based on evolutionary conservation. For instance, mouse CSF-1 cannot bind human CSF1R, yet human CSF-1 can bind and activate mouse CSF1R [59]. In fact, human CSF-1 can activate CSF1R from all species for which it has been tested (human, mouse, feline, sheep, and dog), whereas mouse CSF-1 can activate all nonprimate CSF1R tested (mouse, feline, sheep, and pig) but not human CSF1R [60–66]. The only contact amino acid that is not conserved in mammals is mouse R111, which is Q in humans and varies in other species. Bovine CSF-1 causes growth of murine BMM, presumably through activation of murine CSF1R [61]. Chicken (or at least macrophage growth factor bioactivity in chicken cell-conditioned medium) and feline CSF-1, conversely, are unable to activate the human and mouse CSF1R and are restricted to activating the receptor of their own species [63, 67].

Recent studies identified the CSF-1 genes of several fish species and provided evidence of ancient gene duplications

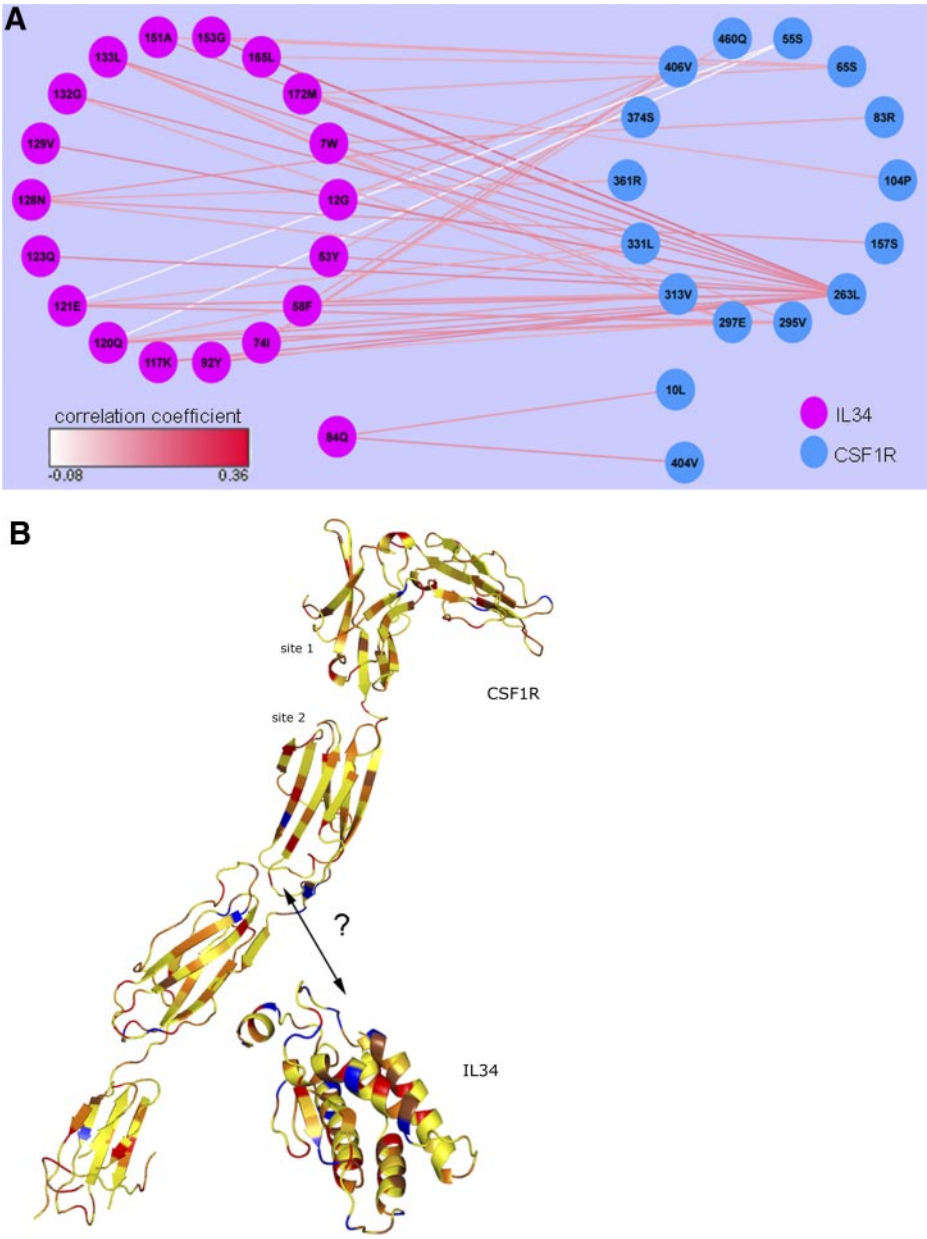

Figure 5. Co-evolution of CSF-1, IL-34, and CSF1R proteins. (A) Diagram summarizing the main results of the co-evolution analyses performed by CAPS, a PERL-based software [37]. The co-evolving residues between IL-34 (left, in pink) and CSF1R extracellular domain (right, in blue) are shown as identified by CAPS [37]. The correlation coefficient is indicated by the color of the line between two co-evolving amino acids and measures the correlated evolutionary variation at these sites. The residue numbers refer to the human sequences. (B) Speculated IL-34-binding mode of CSF1R based on the co-evolution analysis. The ribbons representation of the chicken CSF1R D1–D5 was created by superimposing the chicken CSF1R D1–D3 model produced for Figure 3 and a chicken CSF1R D4–D5 model made using the human KIT D1–D5 (PDB Code 2e9w) as template in 3D-Jigsaw. The chicken IL-34 model is the same as in Figure 2 at a slightly different angle. All of the models were rendered in Polyview-3D. The residues are colored as in Figure 1A, and the co-evolving, homologous residues in chicken are highlighted in blue.

in these species [68]. All piscine CSF-1 peptides have almost complete divergence of the mammalian contact residues, as predicted from the mouse CSF-1/CSF1R co-crystal structure. In the current study, we have identified and expressed the chicken CSF-1 and IL-34 genes and provided evidence that CSF1R is expressed in chicken macrophages, as it is in mammals [and most likely controlled in a similar manner (Fig. 3, C–F)], and that recombinant factors can produce pure macrophage cultures from BM precursors. Hence, the biology of the CSF-1/IL-34/CSF1R triad is also conserved in another class of vertebrates—the birds. Molecular modeling suggests that CSF-1 has a conserved structure in birds and mammals, and in contrast to earlier suggestions [20], we predict that IL-34 also shares that topology characterized by a four-helix bundle [9]. Although there remains the fact that IL-34 lacks all of the cysteines forming the distinctive intrachain disulfide bonds in CSF-1, other growth factors, such as SCF, GM-CSF, and GH, have only one or two intrachain bonds and yet, have that same four-helix bundle topology [9].

It was already known that IL-34 binds CSF1R, and CSF-1 can compete for binding [20], so the finding of a structure similar to that of CSF-1 could have led to the prediction that they were both sharing the same binding sites on CSF1R. This is not compatible with the sequence and predicted structure of IL-34, wherein the contact points identified from the CSF-1/CSF1R co-crystal structure are completely different. The co-evolution study between CSF1R and its ligands performed here revealed a new perspective. When identified on the 3D structure of the proteins, the

co-evolving residue positions uncovered by CAPS are grouped together in distinctive regions. On IL-34, most of them are located at the end distal to the dimer interface, in particular, on a flexible loop between helix C and helix D. None of the co-evolving residues is situated in the corresponding binding site on CSF-1. On CSF1R, the majority of them is positioned at the junction of Domains D3 and D4. If brought together, these two binding sites would fit naturally with each other. Moreover, it is known that the CSF1R D4 domain lacks the characteristic disulfide bond of Ig-like domains that connects the β-sheets and is likely to have greater flexibility [69]. Even the model published recently for CSF1R dimerization and activation could accommodate such an alternative-binding mode for IL-34 [10]. Interestingly, this binding mode is reminiscent of the binding of other four-helix bundle factors (GH, GM-CSF, erythropoietin) to their receptors, involving sites between helices and binding within an intra-domain cleft that is rather closer to the plasma membrane than the D2/3 domain cleft of CSF1R. Activation models of CSF-1/CSF1R suggest that dimerization permits interactions between the two D4 domains, leading to a conformational change that generates signaling [10]. Could IL-34 thereby generate a distinct signal? In the case of the GHR, distinct mutations in the extracellular domain that alter conformation can lead to selective loss of particular signaling pathways in transfected FDCP1 cells [70]. The extracellular domain of CSF1R linked to the intracellular domain of GHR can provide a CSF-1-dependent growth-promoting signal to the same factor-dependent cells (Michael J. Waters, D. A. Hume, unpublished). Therefore, it is conceivable that the two ligands could signal through the same receptor to generate signals that only overlap partly.

The different binding mode of CSF-1 and IL-34 is implied by the fact that although changes of charge in CSF-1-binding sites are matched by changes of opposite charges in the receptor-binding sites, there is no significant correlation coefficient between them. The weak binding of CSF-1 to CSF1R is based on salt bridges and simply requires the presence of opposite charges at the site to occur. This does not have to involve a strict co-evolution of matching amino acids. The binding of IL-34 to CSF1R, however, could be based on hydrogen bonds necessitating specific structural and chemical constraints, thus co-evolving residues.

In addition to suggesting alternative binding sites for IL-34 and CSF1R, the co-evolution study gives us a hint about a functional difference between CSF-1 and IL-34. Indeed, the fact that a correlated evolutionary co-variation with CSF1R can only be detected for IL-34 means that the latter is subjected to stronger selective constraints than CSF-1. In other words, a change in the genetic composition of IL-34 would necessarily involve a reciprocal evolutionary change in the receptor, as the consequences of any loss of activity would be more dramatic than a similar loss for CSF-1. Furthermore, it also suggests that CSF-1 is free to evolve more quickly than IL-34 and without the receptor co-evolving with it. These interpretations, taken together with the greater conservation of IL-34 ($\omega<0.1$ and few predicted sites of positive selection) and the rather different expression pattern of IL-34 compared with CSF-1 (specifically, the lack of expression of the mRNA in cells of the immune system; see www.biogps.org), suggest that IL-34 might perform a more trophic role, regulating functions of macrophages in homeostasis and development as opposed to innate immunity.

Macrophages have many apparent roles in embryonic development [21, 71], but studies of their function in mammals have been constrained by the inaccessibility of the embryo and the fact that CSF-1 is produced by the mother and transmitted across the placenta. We have now identified the key regulators that are likely to control avian myelopoiesis and will be able to take advantage of the accessibility of chicken development in ovo to manipulate expression and function of these genes.

AUTHORSHIP

Valerie Garceau: Cloning of chicken CSF1R and IL-34, expression of the two ligands in HEK, differentiation of BM, bioinformatic analysis (phylogenetic and 3D-structure prediction), authorship; Jacqueline Smith: identification of chicken and zebra finch genes, genetic mapping of CSF-1, positive selection analysis; Ian R. Paton: cloning of chicken CSF1; Megan Davey: ISH; Mario A. Fares: co-evolution analysis and visualization; David P. Sester: supervision and project design; David W. Burt: informatics, identification of chicken and zebra finch genes, genetic mapping of CSF-1; David A. Hume: supervision and project design, informatics, authorship.

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