Identification of Teleost Skin CD8α+ Dendritic-like Cells, Representing a Potential Common Ancestor for Mammalian Cross-Presenting Dendritic Cells

Aitor G. Granja, Esther Leal, Jaime Pignatelli, Rosario Castro, Beatriz Abós, Goshi Kato, Uwe Fischer and Carolina Tafalla

*J Immunol* published online 15 July 2015
http://www.jimmunol.org/content/early/2015/07/15/jimmunol.1500322

---

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2015/07/15/jimmunol.1500322.2.DCSupplemental

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Identification of Teleost Skin CD8α+ Dendritic-like Cells, Representing a Potential Common Ancestor for Mammalian Cross-Presenting Dendritic Cells

Aitor G. Granja,* Esther Leal,* Jaime Pignatelli,* Rosario Castro,* Beatriz Abós,* Goshi Kato,† Uwe Fischer,† and Carolina Tafalla*†

Although fish constitute the most ancient animal group in which an acquired immune system is present, the presence of dendritic cells (DCs) in teleosts has been addressed only briefly, and the identification of a specific DC subset in teleosts remained elusive because of the lack of specific Abs. In mice, DCs expressing CD8α+ in lymphoid tissues have the capacity to cross-present extracellular Ags to T cells through MHC I, similarly to tissue-derived CD103+ DCs and the human CD141+ DC population. In the current study, we identified a large and highly complex subpopulation of leukocytes coexpressing MHC class II and CD8α+. This CD8α+ MHC II+ DC-like subpopulation constituted ∼1.2% of the total leukocyte population in the skin, showing phenotypical and functional characteristics of semimature DCs that seem to locally regulate mucosal immunity and tolerance in a species lacking lymph nodes. Furthermore, we identified trout homologs for CD141 and CD103 and demonstrated that, in trout, this skin CD8α+ DC-like subpopulation expresses both markers. To our knowledge, these results provide the first evidence of a specific DC-like subtype in nonimmune tissue in teleosts and support the hypothesis of a common origin for all mammalian cross-presenting DCs. *The Journal of Immunology, 2015, 195: 000–000.

Dendritic cells (DCs) belong to the family of professional APCs, together with macrophages and B lymphocytes. DCs have a superior capacity to present Ags through MHC class I and II to T lymphocytes and induce their activation and proliferation, consequently triggering CD4+ and CD8+ T cell responses. Together with their role in inducing specific immunity against invading pathogens, mammalian DCs maintain tolerance to self- or innocuous Ags (1).

Although teleost fish constitute the first animal group in which an adaptive immune system is present, a phenotypically distinct DC subtype has not been identified. A few previous studies showed indirect evidence that suggested the presence of DCs in fish. In salmonids, the description of cells containing Birbeck granules (2), cells with DC-like morphology (3), or cells expressing transcripts of DC markers, such as CD209/LAMP3 (4), suggested the presence of DCs. In rainbow trout (Oncorhynchus mykiss), a mammalian protocol was adapted to obtain hematopoietic cultures enriched in cells that showed a greater capacity than B cells or macrophages to stimulate proliferation in an MLR (5). These cells had many characteristic features of mammalian DCs, such as phagocytic capacity, responsiveness to TLR ligands, the expression of DC marker genes (CD83, CD209) or molecules (MHC class II), and migratory capacities. In zebrafish (Danio rerio), a leukocyte subpopulation enriched from lymphoid tissues by affinity to the lectin peanut agglutinin was shown to possess morphological and functional features of mammalian DCs (6). In this same species, enrichment of lymphoid cultures by CD209/DC-SIGN expression defined a cell type that coexpressed MHC class II, CD80/86, and CD83 (7). Despite these previous studies, the identification of a specific DC subset in teleosts remained because of the lack of specific Abs.

In mammals, DC subsets differ in morphology, anatomical location, surface markers, use of transcription factors, and functionality; consequently, this cellular divergence has greatly complicated a complete understanding of their role in the initiation of the adaptive immune response. DCs can be divided into two major subtypes: nonlymphoid tissue-migratory and tissue-resident DCs, sometimes categorized as conventional DCs (cDCs) and plasmacytoid DCs. cDCs are implicated in maintaining tolerance and triggering adaptive immune responses against invading pathogens (1, 8, 9), whereas plasmacytoid DCs are primarily focused on secreting type I IFN in response to viral infections (10). Among cDCs, murine lymphoid tissue–resident DCs include two major subsets defined as CD4+CD8α−CD11b+ and CD8α+CD4−CD11b− DCs, respectively. Recently, an additional CD4−CD8α−CD11b+ DC subtype was described (9). In the same species, CD8α+ DCs congregate in T cell areas of secondary lymphoid tissues, whereas they are absent from nonlymphoid tissues (11) and are considered a nonmigratory DC population derived from blood-borne DC precursors (12) that is specialized in MHC class I presentation and cross-presentation of cell-associated Ags (13).

In contrast, different tissue DC subsets coexist in environmental surfaces, such as the skin. Langerhans cells, the first DC subpopulation described (14), express high levels of langerin, a C-type lectin receptor,
and CD1a (9). Langerin is also expressed in lymphoid CD8α+ DCs and on a subpopulation of tissue DCs that coexpress high levels of CD1033 (15). This CD103+ subpopulation has a high migratory capacity and is able to efficiently cross-present Ags to CD8+ T lymphocytes (16). Recent data suggest that, in mice, both of these cross-presenting DCs, lymphoid CD8α+ DCs and tissue-derived CD103+ DCs, form a common DC lineage. The functionality of these two DC subtypes is dependent on similar transcription factors (17), and they are derived exclusively from pre-DCs under the control of Fli3 ligand, IFN regulatory protein (IRF)8 (18, 19), and Batf3 (20, 21). Additionally, both populations use the CLEC9A lectin to recognize necrotic cells (22) and express the chemokine receptor XCR1 (23). They also express higher levels of TLR3 than do other DC subtypes and, consequently, are responsive to virus-derived intracellular dsRNAs (24). In humans, a discrete CD141+ (BDCA-3 or thombomodulin) DC population in blood also exhibits a cross-presenting function (25). This cell population also expresses CLEC9A (26), XCR1 (23), TLR3 and IRF8 (27), suggesting them as an equivalent of mice cross-presenting cells. In light of these results, some investigators predicted that a CD11c+CD1a+CD141+CLEC9A+ TLR3+ DC subtype should exist in nonlymphoid human tissues (28). In 2012, Haniffa et al. (29) succeeded in identifying this population in human skin, skin-draining lymph nodes, liver, and lung. Through an extensive and detailed phenotypic identification of diverse DC subsets, the investigators demonstrated that human skin CD141+ DCs are closely related to human blood CD141+ DCs and to mice cross-presenting cells (lymphoid CD8α+ DCs and CD103+ migratory DCs) (29). Such a cross-presenting DC subtype in human epithelia was identified as one of the main targets for clinical studies aimed at inducing efficient CD8+ T cell responses against intracellular pathogens and tumors (30).

In the current study, we identified for the first time, to our knowledge, a specific DC-like subpopulation in teleosts that is abundant in the skin, providing the first evidence of a functional dendritic-like cell in a nonlymphoid tissue. These cells did not express transcripts of T cell markers (CD3, TCrx, TCRII, CD8β), but they showed high transcription levels of characteristic DC markers, such as DC-SIGN or LAMP3. Additionally, trout skin CD8α+MHC II+ cells with DC-like morphology were identified in a number of tissues but were found to be most abundant in the skin, providing the first evidence of a functional dendritic-like cell in a nonlymphoid tissue. These cells did not express transcripts of T cell markers (CD3, TCrx, TCRII, CD8β), but they showed high transcription levels of characteristic DC markers, such as DC-SIGN or LAMP3. Additionally, trout skin CD8α+MHC II+ cells were phagocytic for both polystyrene beads and apoptotic cells and were responsive to TLR ligands and to in vivo stimulation, regardless of presenting a semimature profile with high levels of CCR7 surface expression and intermediate levels of CD3 and costimulatory molecules (1). The data presented in the current study lay the foundation for future studies aimed at identifying additional DC markers for the differentiation of other DC subsets in teleosts that would permit functional studies to understand how Ag presentation is regulated in lower vertebrates. Importantly, the identification of CD141 and CD103 expression in this tissue-resident CD8α+ subpopulation that expresses common unique markers of cross-presenting DCs, such as TLR3, Batf3, and IRF8, strongly supports the hypothesis of a common ancestor for vertebrate cross-presenting DCs.

Materials and Methods

Generation and proteomic characterization of an anti-trout MHC class II mAb

Massive immunizations of 6–8-wk-old BALBc mice were performed using large amounts of Ags from rainbow trout head kidney. Animals were immunized i.v. at days 0, 15, 30, and 45. Mice were sacrificed 3 d after the last immunization, and splenocytes were isolated. Generation of hybridomas by fusion of mouse splenocytes with SP2 myeloma cells, isolation of clones, and purification of specific anti-trout mAbs were performed as previously described (31). Rainbow trout head kidney protein lysates were used to test the specificity of specific Abs. The clone 1A1-5C10 mAb recognized a protein ~30 kDa in Western blotting (Supplemental Fig. 1A) and immunoprecipitation assays (Supplemental Fig. 1B), which were performed as previously described (32). For the identification of such Ag, a similar immunoprecipitation was performed, and the 30-kDa band from silver-stained gels was excised manually, deproteinized in 96-well plates, and processed automatically in a PROTEINER DP (Bruker Daltonics). The digestion protocol used was described previously (33). Digested peptides of each sample were subjected to one-dimensional nano-liquid chromatography–electrospray ionization/multistage mass spectrometry analysis using a nano-electrospray ionization system (Eksigent Technologies, AB Sciex) coupled with a high-speed TripleTOF 5600 mass spectrometer (AB Sciex) with a MaxEnt Ion Source. The analytical column used was a silica-based (C18) reversed-phase column C18 ChromXP 75 μm x 15 cm, with 3 μm particle size and 120 Å pore size (AB Sciex), which was switched on-line with a C18 ChromXP trap column. Mass spectrometry and multistage mass spectrometry data were acquired using information-dependent acquisition mode with Analyst TF 1.5.1 Software (AB Sciex). For information-dependent acquisition parameters, a 0.25-s MS survey scan in the mass range of 350–1250 Da was followed by 35 MS/MS scans of 100 ms in the mass range of 100–1800 (total cycle time: 3.8 s). Raw data file–conversion tools generated mgf files that also were searched against the National Center for Biotechnology Information database with “Onchorhyncus mykiss + Salmo salar + Mus musculus” taxonomy restriction, containing 530,414 protein-coding genes, using Mascot Server version 2.5 (Matrix Science). Search parameters were set as follows: carboxymethyl (C) as fixed modification and oxidation (M) as variable modification. Peptide mass tolerance was set to 25 ppm and 0.05 Da for fragment masses, and one missed cleavage was allowed. Only the peptides with an individual molecular weight search score ≥ 20 were considered correctly identified.

Experimental fish

Female rainbow trout (O. mykiss) ~ 50 g were obtained from Centro de Acuicultura El Molino (Madrid, Spain) and maintained at the animal facilities of the Centro de Investigación en Sanidad Animal in an aerated-recirculating water system at 16°C, with a 12:12-h light/dark photoperiod. Fish were fed twice a day with commercial food (Skretting) and were acclimatized to laboratory conditions for ~2 wk prior to any experimental procedures. All of the experiments described comply with the Guidelines of the European Union Council (2010/63/EU) for the use of laboratory animals and were approved by the Instituto Nacional de Investigación Agraria y Alimentaria Ethics Committee.

Tissue sampling

Rainbow trout were killed by MS-222 (Sigma) overdose, and blood was extracted with a heparinized needle from the caudal vein and diluted 10 times with Leibovitz medium (L-15; Invitrogen) supplemented with 100 U/ml penicillin together with 100 μg/ml streptomycin (Life Technologies), 10 U/ml heparin (Sigma), and 5% FCS (Life Technologies). Spleen, head kidney, thymus, intestine, gills, and skin were collected. Single-cell suspensions from spleen, head kidney, gills, and thymus were obtained using 100-μm nylon cell strainers (BD Biosciences). The intestines were opened lengthwise, washed in PBS, and cut into small pieces. The skin was cut off the fish with a scalpel, muscle tissue was removed, and the skin was cleaned with ice-cold PBS and cut into small pieces. For both tissues, the cell-extraction procedure started with 30 min agitation at 4°C in L-15 medium with penicillin and streptomycin and 5% FCS, followed by agitation in PBS with 1 mM EDTA and 1 mM DTT for 30 min. Finally, the tissues were digested with 0.15 mg/ml collagenase (Sigma) in L-15 for 1.5 h at 20°C. All cell suspensions were placed onto 30/51% discontinuous Percoll (GE Healthcare) density gradients and centrifuged at 500 × g for 30 min at 4°C. The interface cells were collected and washed twice in L-15 containing 5% FCS. When required, cells were treated with LPS (100 μg/ml), polyniosinic-polycytidylic acid (poly I:C; 50 μg/ml), or TNF hapten conjugated to the keyhole limpet hemocyanin carrier protein (TNP–KLH; 5 μg/ml) for 16 h.

Flow cytometry

The anti-trout CD8α (mAb rat IgG3; 7 μg/ml) and the anti-trout CCR7 (pAb rabbit IgG; 2 μg/ml) used in this study were characterized previously (34, 35). The anti-trout MHC class II-chain (mAb mouse IgG1; 2 μg/ml) was characterized above. Primary cells were incubated for 30 min with primary Abs, washed twice with staining buffer (PBS containing 1% FCS and 0.5% sodium azide), and stained for 20 min with secondary Abs for anti-CD8α.
[R-PE F(ab′)_2], fragment of goat anti-rat IgG (H+L); Life Technologies] and anti-CCR7 [Alexa Fluor 488 F(ab′)_2], fragment of goat-antirabbit IgG (H+L); Life Technologies], when needed. An Alexa Fluor 647 fluorescently-labeled version of MHC class II Ab was used for flow cytometry (previously conjugated using the Alexa Fluor 647 Protein Labeling Kit; Life Technologies). After incubation, cells were washed three times with staining buffer and analyzed on a FACScalibur flow cytometer (BD Biosciences) equipped with CellQuest Pro software. Cell populations were sorted by flow cytometry on a FACSAria III (BD Biosciences), using their forward scatter (FSC)/side scatter (SSC) and fluorescence characteristics. In all cases, isotype controls for mouse mAbs, rat mAb, and rabbit polyclonal Ab (BD Biosciences) were tested in parallel to discard unspecific binding of the Abs. Flow cytometry analysis was performed with FlowJo 10 (TreeStar).

Transcriptional studies
Total cellular RNA was isolated from trout spleen- and skin-sorted populations or the RTS11 (rainbow trout macrophages) cell line (36) using the Power SYBR Green Cells-to-CT Kit (Invitrogen), following the manufacturer’s instructions. RNA was treated with DNase during the process to remove genomic DNA that might interfere with the PCR reactions. Reverse transcription also was performed using the Power SYBR Green Cells-to-CT Kit (Invitrogen), following the manufacturer’s instructions. To evaluate the levels of transcription of the different genes, real-time PCR was performed with a LightCycler 96 System (Roche) using SYBR Green PCR Master Mix (Applied Biosystems) and specific primers (Supplemental Fig. 2). Each sample was measured in triplicate under the following conditions: 10 min at 95°C, followed by 40 amplification cycles (15 s at 95°C and 1 min at 60°C). A melting curve for each PCR was determined by reading fluorescence at every degree between 60 and 95°C to ensure that only a single product had been amplified. The expression of individual genes was normalized to the relative expression of trout housekeeping gene EF-1α, elongation factor, and the expression levels were calculated using the 2^{ΔΔCt} method, where ΔCt is determined by subtracting the EF-1α value from the target Ct. No template negative controls were included in all of the experiments.

Phagocytic activity
For the analysis of bead phagocytosis, skin leukocytes were seeded in 24-well plates (Nunc) at a cell density of 1 × 10^6 cells/well and incubated for 16 h at 20°C with fluorescent beads (FluoSpheres Microspheres, 1.0 μm, Crimson Red Fluorescent 625/645, 2% solids; Life Technologies) at a cell/bead ratio of 1:10 or without beads as negative controls. Cells were harvested after a standard cell scraper (Corning). Noningested beads were removed by centrifugation (100 × g for 10 min at 4°C) over a cushion of 3% (w/v) BSA (Fraction V; Fisher Scientific) in PBS supplemented with 4.5% (w/v) n-glucose (Sigma). Cells were resuspended in staining buffer, labeled with the flow cytometry Abs specified for each assay, and analyzed on a FACScalibur flow cytometer equipped with CellQuest software (BD Biosciences).

For the analysis of phagocytosis of apoptotic/dead cells, single-cell suspensions from spleen were obtained as described above, and the resulting splenocytes were labeled with CellTrace Far Red stain (Life Technologies) at a final concentration of 1 μM, following the manufacturer’s instructions. Labeled cells were incubated for 16 h at 20°C in the presence of 1 μM A23187 calcium ionophore to induce apoptosis, as verified by >90% Annexin V-positive staining of splenocytes. Then, fluorescently labeled apoptotic splenocytes were added to skin leukocytes or RTS11 macrophages disposed into 24-well plates (Nunc) at a density of 1 × 10^6 cells/well and a effector/apoptotic cell ratio of 1:10. Plates were incubated for 16 h at 20°C. Cells were harvested using a standard cell scraper (Corning) and resuspended in staining buffer. For flow cytometric analysis, three samples were stained for “DSOs,” CellTrace Far Red and CellTrace Far Red+ RTS11 macrophages were calculated by subtracting the frequency of positive events at 2°C (binding) from the frequency at 20°C (uptake).

In vivo stimulations
Rainbow trout were nonlethally challenged with viral hemorrhagic septicaemia virus (VHSV) through bath infection. Briefly, groups of three fish ~50 g were transferred to 2 l viral solution containing 5 × 10^4 TCID_{50}/ml VHSV, strain 0771. After 1 h of viral adsorption at 16°C, fish were transferred to their original tanks (20 l) and maintained for 16 h. In other experiments, groups of three rainbow trout were maintained in 10-l tanks and exposed to zymosan particles in the water at a final concentration of 0.01% (w/v) for 16 h. After treatment, fish were sacrificed by overdose perfusion to MS-222. Skin was sampled and processed for flow cytometry analysis and for extraction of RNA for subsequent gene expression analysis. Mock-treated groups maintained under the same experimental conditions were included in the VHSV and zymosan experiments.

Immunofluorescence assay
Skin leukocyte suspensions obtained from rainbow trout of clone C25 were used for the immunofluorescence analysis of CD8+ MHC II+ cells. The separated leukocytes were seeded on a polystyrene–coated slide and incubated at 16°C for 30 min. After gently washing with the culture medium, the slides were dried and fixed in ice-cold acetone for 5 min. Fixed cell slides were incubated with mAbs against trout CD8α and MHC class II diluted with the culture medium for 1 h at 4°C. Slides were washed with the fixed medium and mounted with an antifade (H+L) Alexa Fluor 488 conjugate and anti-mouse IgG1 Alexa Fluor 568 (both from Life Technologies) for 30 min at 4°C. Slides were counterstained with Hoechst 33342 (Life Technologies) and examined with an ECLIPSE Ti-S Inverted Research Microscope (Nikon, Tokyo, Japan). Digital images were captured using NIS-Elements BR 3.2 software (Nikon).

Immunohistochemistry and confocal microscopy
Rainbow trout skin from anesthetized and exsanguinated rainbow trout was embedded in PolyFreeze cryostat mounting medium (Sigma), immediately snap-frozen in liquid nitrogen, and stored at −80°C until used. Cryostat sections with a thickness of 20 μm were prepared using a Leica CM3050 microtome and mounted on SuperFrost glass slides (Menzel-Gläser). Dry sections were fixed in acetone for 10 min, air-dried, encircled with a hydrophobic compound (ImmunoPen; Calbiochem), incubated for 1 h at room temperature with a blocking solution (TBS buffer [pH 7.5], containing 0.1% BSA, 0.02% Tween-20, and 10% rabbit and goat serum), and stained with Abs against trout MHC class II β-chain (Alexa Fluor 647 fluorescently-labeled version of MHC class II Ab) and trout CD8α (mAb rat IgG). Samples were washed and incubated with the CD8α secondary Ab Alexa Fluor 488 F(ab′)2 fragment of goat anti-rat IgG (H+L) (Life Technologies). Samples were counterstained with 1 μg/ml DAPI (Sigma).

Scanning electron microscopy
Sorted skin CD8+ MHC II+ cells from the myeloid gate were plated on polystyrene–coated cover glasses before being fixed with 2.5% (v/v) paraformaldehyde in PBS for 10 min at room temperature. After three washes in PBS, cells were subjected to sequential dehydration with gradient ethanol (50, 70, 90, and 100%) and stored at −80°C until used. Cryostat sections, followed by isoamyl acetate treatment (three times, 5 min each), were dried and coated with gold, and a scanning electron microscope (JEOL JSM-7600F) was used for observation.

MLR
Skin CD8+ dendritic-like cells were isolated by cell sorting, as described above. The cells were cultured for 12 h in L-15 medium supplemented with 5% FCS in the absence or the presence of the T-cell–dependent Ag TNP–KLH. Because there are no Abs against extracellular pan-T cell markers available for rainbow trout, we used T cell–enriched cultures as responder cells. These T cell–enriched cultures from isogeneic and allogeneic skin leukocytes were obtained by depletion of splenocytes labeled with anti-IgM, anti-MHC class II mAbs and mAbs specific for thrombocytes (37) and myeloid cells (38), using flow sorting. The resulting negative population, representing 10% of splenocytes, was labeled with CellTrace Far Red (Life Technologies). The enrichment in T cells was assessed by intracellular staining with an anti-CD3 Ab (39), which showed >90% of CD3+ cells in the cultures. DCs were cocultured with isogeneic (auto-MLR) or allogeneic (allo-MLR) T cell–enriched splenocytes at a 1:50 DC/splenocyte ratio. After 24 h, cells were harvested, and total RNA was isolated for gene expression analysis, as described above. In parallel, 4 d after the MLRs were set up, cocultured samples were analyzed by flow cytometry to measure cell proliferation through the degree of activation of the CellTrace Far Red vital marker.

Identification of the CD141 gene in rainbow trout
The human CD141 protein sequence was used as a query against expressed sequence tag (EST) databases from rainbow trout (O. mykiss) in the
FIGURE 1. Distribution of CD8α⁺MHC II⁺ cells among leukocytes from different organs. (A) Flow cytometry analysis of rainbow trout leukocytes isolated from trout tissues (spleen, head kidney, PBLs, thymus, gut, gills, and skin) and stained with anti-CD8α and anti-MHC class II β-chain mAbs. For each individual tissue, FSC/SSC profiles are shown (left panels) and gates for lymphoid and myeloid cells were defined. Two-color CD8/MHC class II dot plots of gated cells are also shown (right panels). Percentage of CD8α⁺MHC II⁺ cells among the total number of cells is shown in the upper right corner. (B) Mean (± SD) percentages of CD8α⁺MHC II⁺ cells from three independent experiments (n = 9 fish). (C) The levels of transcription of several immune markers known in rainbow trout were analyzed by real-time PCR. Myeloid CD8α⁺MHC II⁺ cells from spleen and skin were isolated by cell sorting, and RNA was obtained. The relative expression of the indicated genes in relation to the endogenous control EF-1α was calculated for each sample, and the mean values (± SD) from three independent experiments, containing three animals/experiment, were calculated.
Identification of CD8+ cells. Arrowheads show double positive cells. Scale bars, 20 μm.

**FIGURE 2.** Location, morphology and gene expression of skin CD8+ MHC II+ cells. (A) For immunofluorescence analysis, cryostat sections with a thickness of 20 μm were prepared from rainbow trout skin, fixed, and labeled with anti-CD8α (red) and anti-MHC class II (green) Abs, counterstained with Hoechst (blue), and analyzed by confocal microscopy. Arrowheads show double positive cells. Scale bars, 20 μm. (B) For identification of CD8α+MHC II+ cells, total leukocytes from skin were fixed and labeled with anti-CD8α (green) and anti-MHC class II (red) Abs, counterstained with Hoechst (blue), and analyzed by confocal microscopy. Scale bars, 5 μm. (C) CD8α+MHC II+ cells from skin were isolated by cell sorting and then incubated onto poly-L-lysine–treated glass slides, fixed, mounted, and analyzed by light microscopy. Scale bar, 5 μm. (D) Myeloid CD8α+MHC II+ cells from skin also were analyzed using a scanning electron microscope. Scale bar, 5 μm. (E) Sorted myeloid skin CD8α+MHC II+ cells were analyzed by real time-PCR. Relative expression of the indicated genes to the endogenous control EF-1α was calculated for each sample, and mean (± SD) values from three independent experiments, including three fish per experiment, were obtained.

National Center for Biotechnology Information’s database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using tBLASTn searches. A rainbow trout EST that encoded a CD141-like sequence was identified (accession number CA377463). The sequence lacked a stop codon; therefore, 3′ RACE was performed to obtain the complete sequence using cDNA from PBLs, a 3′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen), and primers CD141-3′ RACE (5′-GGTGATGGTGTTGAAG-GATGCGCTATGGC-3′) and CD141-3′ RACE nested (5′-GGGCTACA-CATCCCTGTTGAAAAGAAGG-3′). An overlapping fragment that contained the final segment of the CD141 coding sequence and the 3′ untranslated region was amplified. Primers were then designed to amplify the full coding sequence, which was again sequenced. The complete CD141 nucleotide sequence was analyzed within the ExPASy Molecular Biology server (http://us.expasy.org) and deposited in GenBank under accession number KP203844 (http://www.ncbi.nlm.nih.gov/nuccore/KP203844).

**Phylogenetic analysis**

Phylogenetic analyses of the CD141 and CD103 protein sequences were performed using MegAlign Software (DNASTAR, Madison, WI) and the Clustal V algorithm. Statistical parameters of phylogenetic trees were determined by bootstrap analysis using 1000 replicates.

**Statistical analysis**

Statistical analyses were performed using a two-tailed Student t test with the Welch correction when the F test indicated that the variances of both groups differed significantly. The differences between the mean values were considered significant on different degrees, where *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.005 (GraphPad Prism 4 software).

**Results**

CD8α+ cells that express MHC class II are present in all rainbow trout tissues

Through the combined use of an anti–MHC class II β-chain and an anti-CD8α Ab in flow cytometry, we observed that a considerable percentage of CD8α+ cells expressed MHC class II on the cell membrane (Fig. 1A). These double-positive CD8α+ MHC II+ cells were present in all tissues examined, including spleen, head kidney (main hematopoietic organ), thymus, gut, gills, and skin, whereas only a few were detected in blood. Because CD8α+ cytotoxic T cells are not expected to express MHC class II on the cell membrane (40), and, in contrast, certain mammalian DC subsets are double positive for CD8α and MHC class II (11), our results suggested the possibility of these cells being DCs. Cells were analyzed according to their FSC/SSC profile (Fig. 1A), including cells within the lymphoid gate (FSClowSSc(low)) representing lymphocytes and thrombocytes (the latter being nucleated in teleosts) or within the myeloid gate (FSC(high)SSc(low/high)), in which larger cells, such as macrophages and neutrophils, are commonly found (41). Because of their size and morphology, DCs would be expected to appear in the myeloid gate. However, two types of CD8α+ MHC II+ cells were found in all tissues examined: one population with lymphoid morphology and another one with myeloid morphology. Although the thymus was the organ in which the highest percentages of lymphoid CD8α+ MHC II+ cells were identified (3.11% of total leukocytes), CD8α+ MHC II+ cells within the myeloid gate were most abundant in skin (1.16%), followed by the gills (0.67%), the thymus (0.65%), and the gut (0.3%) (Fig. 1B). Strikingly, secondary lymphoid tissues, such as spleen and head kidney, harbor <0.1% and 0.25%, respectively, CD8α+ MHC II+ cells within the myeloid gate, suggesting that these double-positive cells are preferentially located in peripheral mucosal tissues in teleosts.
Transcriptional profile of spleen and skin CD8α-MHC II+ cells

Seeking further evidence that CD8α-MHC II+ cells were DCs, we sorted spleen and skin CD8α-MHC II+ cells within the myeloid gate and skin CD8α-MHC II+ cells from the lymphoid gate following the gating strategy described in Supplemental Fig. 3A and 3B. Sorted cells were analyzed on their expression of different signature genes through real-time PCR. Skin myeloid CD8α-MHC II+ cells showed no CD3, TCR-α, TCR-γ, or CD8β transcription, ruling out the possibility of them being cytotoxic T cells (Fig. 1C). In concordance with their staining patterns, skin myeloid CD8α-MHC II+ cells expressed CD8α and MHC class II transcripts. Furthermore, these cells also transcribed DC-SIGN and CD11b (42). Interestingly, skin myeloid CD8α-MHC II+ cells also transcribed IRF8 and Batf3, which are essential for the development of mammalian cross-presenting DCs (18–21), but not IRF4, which is implicated in the development of other CD8+ DC subtypes in mammals (43). Compared with skin CD8α-MHC II+ cells, a different transcription profile was recorded for splenic myeloid CD8α-MHC II+ cells, showing significant CD8β, CD3, and TCR-α mRNA expression levels, suggesting the presence of mixed cell populations (Fig. 1C). Although no TCR transcripts were detected in skin CD8α-MHC II+ cells from the lymphoid gate, CD3 transcripts were found (Supplemental Fig. 3C). Consequently, further research needs to be undertaken to clarify the nature of these populations. When the transcriptional profile of skin CD8α-MHC II+ cells from the myeloid gate was compared with that of CD8α+ cytotoxic T cells from spleen (CD8α+ MHC II+ cells from the lymphoid gate), we verified that skin CD8α-MHC II+ cells had a distinct DC-like profile (Supplemental Fig. 4). CD8α+ cytotoxic T cells expressed typical signature transcripts (i.e., CD3, TCR-α, TCR-γ, CD8α, CD8β, and MHC class I), whereas they were unable to transcribe other DC markers, such as MHC class II or DC-SIGN. In contrast, to rule out the possibility of these cells being macrophages, their transcriptional profile was compared with that of rainbow trout macrophages from the RTS11 cell line. Although macrophages transcribed MHC class I, MHC class II, and DC-SIGN as expected, they did not transcribe CD8α (Supplemental Fig. 4), in agreement with the fact that they do not express CD8α protein on the cell surface (data not shown) or Batf3. Overall, our transcriptional results strongly support that skin CD8α-MHC II+ cells within the myeloid gate correspond to a subset of teleost DCs, and our subsequent experiments focused exclusively on this DC-like subpopulation.

The location of CD8α-MHC II+ cells was analyzed by immunofluorescence of trout skin sections. We observed dispersed myeloid CD8α-MHC II+ cells located primarily in the epidermal layer (Fig. 2A). Moreover, when we analyzed skin leukocytes by fluorescence microscopy, we observed round globular CD8α+ MHC II+ cells (Fig. 2B). To further characterize the morphology of these cells, they were sorted by flow cytometry and analyzed by

**FIGURE 3.** Phagocytic capacities of skin CD8α cells. Leukocytes from trout skin were incubated with Crimson Red fluorescent polystyrene beads (1 μm diameter) at a ratio of 1:10 (cell/beads) for 16 h and then centrifuged through a 3% BSA + 4.5% glucose gradient to remove adhered beads. (A) Cells were stained with mAbs to CD8α and analyzed by flow cytometry. Lymphoid cells (upper panels) and myeloid cells (lower panels) were gated, and CD8α and CD8α+ cells were further selected to analyze and measure the fluorescence of internalized beads (line graphs). The percentages of cells containing beads were determined using control samples without beads to establish the phagocytosis gate shown in the line graphs. Average percentage of phagocytic CD8α and CD8α+ cells (B) and median fluorescence intensity of the beads (C) were used to determine the phagocytic capacity of each cell type. Data are representative of nine individual fish from three independent experiments. (D) Giemsa staining of CD8α+MHC II+ skin cells after incubation with zymosan particles for 3 h (zymosan) compared with the same cell type after incubation with control medium. Scale bar, 5 μm. (E) Skin leukocytes or RTS11 trout macrophages were incubated with labeled apoptotic splenocytes at a 1:1 ratio (effector/apoptotic cells), as described in Materials and Methods. Uptake of apoptotic cells by skin CD8α+ DCs was analyzed by flow cytometry to determine the percentage of CellTrace Far Red+ CD8α+ cells after subtracting the frequency of positive events at 2°C (binding; filled graph) from the frequency at 20°C (uptake; open graph) (left panel). Uptake of apoptotic cells by RTS11 macrophages was analyzed in parallel. A representative bar graph shows uptake of apoptotic cells by skin CD8α+ cells and the percentage of phagocytic cells obtained in skin leukocytes and macrophages (n = 9 fish, mean ± SD). ***p ≤ 0.005.
light microscopy (Fig. 2C) and scanning electron microscopy (Fig. 2D). Cells showed a round irregular morphology with small membrane projections, as described previously for the corresponding subpopulation of DCs in mammals (44). Interestingly, further transcriptional analysis of skin myeloid CD8α+MHC II+ cells revealed that they showed an activated phenotype with extremely high CCR7, CXCR4, IFN-γ, BAFF, LAMP-3, and CD40 transcription levels and intermediate levels of CD83 (Fig. 2E). Additionally, skin CD8α+MHC II+ cells transcribed MHC class I–associated molecules, showing high levels of tapasin and calreticulin and intermediate levels of Erp57 (Fig. 2E). Moreover, skin CD8α+MHC II+ cells transcribed cathepsin Z and CD80/86, known to be associated with MHC class II–dependent Ag presentation in mammals. This last molecule, with similar homologies to mammalian CD80 and CD86, previously was designated CD80/86 (45). These results reveal that skin CD8α+MHC II+ cells have the essential machinery for both MHC class I—and MHC class II–mediated Ag presentation. The transcriptional profile, together with the morphological characterization, led us to conclude that these skin CD8α+MHC II+ cells are, in fact, a teleost DC-like subpopulation.

Phagocytic capacity of teleost skin CD8α+MHC II+ cells

Although it was widely assumed that activated DCs lose their ability to phagocytose, Platt et al. (46) demonstrated that mature DCs retain their capacity to capture, process, and present Ags internalized via endocytic receptors, whereas only their capacity to execute micropinocytosis is downregulated through maturation. Trout skin CD8α+MHC II+ cells showed a high phagocytic capacity, in contrast to lymphoid CD8α+ cells that were not phagocytic at all (Fig. 3A, 3B). After 16 h of incubation with 1 μm Crimson red–labeled polystyrene beads, ~53% of the CD8α+MHC II+ cells had internalized beads, whereas only ~5% of CD8α+ cells within the myeloid gate had internalized beads (Fig. 3A, 3B). Additionally, the median fluorescence intensity in CD8α+MHC II+ cells was significantly higher than that observed in CD8α+ cells within the myeloid gate (Fig. 3C), indicating that the average number of particles internalized per cell was higher for CD8α+ MHC II+ cells than for CD8α+ MHC II+ cells. Sorted CD8α+MHC II+ cells were also capable of internalizing different types of particulate Ags, such as zymosan (Fig. 3D). These results strengthen our findings that trout skin CD8α+MHC II+ cells within the myeloid gate are, in fact, phagocytic CD8α+ DCs.

A distinctive feature of mammalian cross-presenting DCs is their superior capacity to uptake apoptotic and/or dead cells (22); thus, we also compared the capacity of trout skin CD8α+MHC II+ cells and trout RTS11 macrophages to phagocytose apoptotic cells. As shown in Fig. 3E, skin dendritic-like cells were capable of uptaking apoptotic splenocytes, whereas macrophages were not.

Response through TLRs

The pattern of TLR expression is commonly used in mammals to characterize DC subtypes. Thus, we examined the transcription levels of TLRs in skin CD8α+MHC II+ cells, including all known trout TLR genes (i.e., TLR1, TLR2, TLR3, TLR5, TLR7, TLR8α and TLR9), together with TLR22, a fish-specific cell surface sensor of dsRNA (47). Interestingly, trout skin CD8α+MHC II+ cells transcribed high levels of TLR1, TLR5, TLR7, TLR8α, TLR9, and TLR22, intermediate levels of TLR3, and very low levels of TLR2 (Fig. 4A). Although murine CD8α+ DCs do not express TLR7 (48), TLR3 is considered a signature marker for cross-presenting DCs, murine CD103+ tissue and CD8α+ lymphoid DCs, and the human CD141+ DC lineage (29). Consequently, we analyzed whether trout skin sorted CD8α+ dendritic-like cells responded to stimulation with poly I:C, a known ligand for teleost TLR3 and TLR22 (49), or bacterial LPS, as an irrelevant TLR3 ligand. We found that poly I:C provoked a significant upregulation of CCR7, BAFF, IFN-γ, MHC class I, MHC class II, and CD83 in the skin CD8α+ DC-like subpopulation, whereas no significant changes in gene expression were found after incubation with LPS (Fig. 4B). These results demonstrate that, similarly to mammalian cross-presenting DCs, trout skin CD8α+ dendritic-like cells are responsive to TLR3 ligands.

Teloskin skin CD8α+MHC II+ cells express CD141 (BDCA-3/CD40) and CD103 (integrin αE)

Multiple lines of evidence point to a common cross-presenting DC lineage for mice lymphoid CD8α+ DCs and tissue-migratory CD103+ DCs (17–19, 22–24). In humans, blood CD141+ cells also cross-present Ags and exhibit markers in common with mice CD8α+ DCs (23, 25–27), suggesting a common origin for all mammalian cross-presenting cells (29). To investigate whether such an ancestor was present in an ancient vertebrate, such as rainbow trout, we searched the databases for trout CD141 (also designated as BCDA3 or thrombomodulin) and CD103 (also designated as integrin α E) sequences to determine whether trout skin CD8α+MHC II+ cells express these markers.

Using the human CD141 protein sequence, we searched within trout EST databases and identified a sequence with close homology to CD141 (GenBank accession number CA377463.1). This sequence lacked the 3′ end; thus, we conducted 3′ RACE to obtain the full coding sequence. Posterior phylogenetic analysis through neighbor joining of the predicted protein with different mamma-
lian and fish thrombomodulin sequences confirmed this identification (Fig. 5A). CD141 transcription was detected at extremely high levels in trout skin sorted CD8+ dendritic-like cells, whereas it was not expressed in CD8α+MHC II+ cells from the lymphoid gate or in spleen cytotoxic T cells identified as lymphoid CD8α+ MHC II+ cells (Fig. 5B).

Similarly, we searched the rainbow trout EST databases for a CD103 homolog using the human CD103 sequence as a template. In this case, we found a sequence with high homology to human CD103 that contained a full coding region (GenBank accession number CDQ67442.1). Further phylogenetic analysis confirmed that this sequence was, in fact, a rainbow trout CD103 homolog (Fig. 5C). Interestingly, trout skin sorted CD8α+MHC II+ cells also transcribed CD103 (Fig. 5D). CD8α+MHC II+ cells from the lymphoid gate and sclenic cytotoxic T cells also transcribed CD103, but with lower expression levels. The fact that skin DCs from an ancient vertebrate, such as the rainbow trout, coexpress CD8α, CD141, and CD103 strongly supports the existence of a common origin for all mammalian cross-presenting DCs.

**Teleost skin CD8α+MHC II+ cells prime T cells**

One of the main defining features of DCs is their capacity to stimulate T cells. To demonstrate their T cell–activating potential, we performed an MLR. For this, we sorted skin CD8α+MHC II+ cells and incubated them overnight in the presence of TNP–KLH (a T cell–dependent Ag) or left them unstimulated. This CD8+ DC-like subpopulation was coincubated with isogeneic (auto-MLR) or allogeneic (allo-MLR) splenocyte cultures enriched in T cells from which all APCs had been depleted. As expected, the addition of CD8α+ dendritic-like cells to the T cell–enriched splenocyte cultures significantly induced cell proliferation in isogeneic and allogeneic cultures (Fig. 6A, 6B). The fact that dendritic-like cells were previously incubated with TNP–KLH had no effect on their T cell–activating capacities (Fig. 6A, 6B), pointing to an activated state of skin-resident CD8α+ dendritic-like cells. When transcriptional analyses were performed, we observed that T-bet mRNA levels were increased significantly in auto-MLRs, whereas Eomes mRNA levels were significantly higher in auto-MLRs in which dendritic-like cells were pretreated with TNP–KLH and in allo-MLRs performed with unstimulated dendritic-like cells (Fig. 6C). Additionally, FoxP3 mRNA levels were higher in both auto-MLR and allo-MLR cultures in which dendritic-like cells were not stimulated with TNP–KLH. Finally, neither perforin nor CD40L mRNA levels were induced by CD8α+ dendritic-like cells. Altogether, these results demonstrate a capacity for the teleost skin CD8α+ DC-like subpopulation to activate T lymphocytes exhibiting both stimulatory and regulatory phenotypes.

**Skin CD8α+MHC II+ cells express high levels of CCR7 on their cell surface**

In mammals, CCR7 governs DC migration from the skin to the lymphatic vessels in steady-state conditions, playing an important role in the development and function of naive T cells. In teleosts, however, the expression of CCR7 on skin-resident DCs has not been investigated.

---

**FIGURE 5.** CD141 (BDCA-3) and CD103 (integrin αE) are present in rainbow trout and expressed in the CD8α+ DC-like subpopulation. Phylogenetic analyses of the CD141 (A) and CD103 (C) protein sequences were performed using MegAlign Software by comparison using the Clustal V algorithm and the neighbor-joining method. Statistical parameters of phylogenetic trees were determined by bootstrap analysis using 1000 replicates. Bootstrap values are shown above the branches. Skin CD8α+MHC II+ cells from the lymphoid gate and skin CD8α+MHC II+ cells (myeloid gate), as well as spleen CD8α+ T cells (CD8α+MHC II+ events from the spleen lymphoid gate) were isolated by cell sorting, and RNA was obtained. The relative expression of CD141 (B) and CD103 (D) to the endogenous control EF-1α was calculated for each sample, and mean (± SD) values from three independent experiments, involving three animals/experiment, were calculated.
role in the maintenance of peripheral tolerance (50). Using a recently described anti-trout CCR7 polyclonal Ab (35), we evaluated the levels of CCR7 surface expression in skin CD8<sup>a</sup> +MHC II+ cells. Because CCR7 is now known to be highly expressed in thymocytes (Fig. 7A, 7B) (51), skin expression of CCR7 was compared with the corresponding levels in thymic CD8<sup>a</sup> +MHC II+ cells (Fig. 7C). In trout skin, the percentages of myeloid cells with membrane-bound CCR7 were very high no matter whether they were CD8<sup>a</sup> +MHC II+ cells or not (Fig. 7A, 7B). However, a significantly higher CCR7 median fluorescence in the CD8<sup>a</sup> DC-like subpopulation indicates that the density of CCR7 molecules on the cell surface is highest in skin CD8<sup>a</sup> MHC II+ cells (Fig. 7B). Similar cell surface expression patterns were recorded in thymocytes (Fig. 7D).

**In vivo exposure to zymosan activates the skin CD8<sup>a</sup> DC-like subpopulation**

To understand whether skin CD8<sup>a</sup> MHC II+ cells were responsive to in vivo stimulation, we bath exposed fish to zymosan or VHSV and analyzed its effect on the number of CD8<sup>a</sup> dendritic-like cells in the skin and in the transcriptional regulation of different DC activation markers. A significant increase in the percentage of skin CD8<sup>a</sup> dendritic-like cells was observed in fish exposed to zymosan (Fig. 8A) or infected with VHSV (Fig. 8B) in comparison with nonstimulated fish. This increase in the number of CD8<sup>a</sup> dendritic-like cells in skin was accompanied by a significant increase in the transcription levels of BAFF, MHC class II, CD83, DC-SIGN, tapasin, and calreticulin in CD8<sup>a</sup> dendritic-like cells from both zymosan-treated (Fig. 8C) and virus-infected fish (Fig. 8D). In contrast, MHC class I and CD40 expression was significantly upregulated only after virus infection (Fig. 8D) and not by zymosan treatment (Fig. 8C). In contrast, ERp57 transcription was only upregulated by zymosan treatment (Fig. 8C). Additionally, transcription levels of IFN-γ were augmented in CD8<sup>a</sup> dendritic-like cells from both zymosan (Fig. 8C) and VHSV-treated (Fig. 8D) animals, although the increase was considerably higher in virus-infected fish, indicating antiviral responses of fish DCs.
Jawed fish are the first evolutionary group in which adaptive immune responses are present. This is not a general rule for fish species, because no MHC class II and CD4 genes were found in the Atlantic cod genome (52). However, most fish species investigated possess these molecules; consequently, it would be expected that all of the elements implicated in Ag processing and presentation also exist in this animal group. Although there is evidence for the existence of DCs in teleosts, the lack of adequate immunological tools has hampered a full phenotypic and functional characterization of DC lineages, and only a few studies have analyzed the properties of DC-like populations following enrichment protocols (5–7). In the current work, we identified, in trout, a large and highly complex subpopulation of leukocytes, of irregular shape and coexpressing MHC class II and CD8\(^+\), which resemble mammalian CD8\(^+\) DCs. In the skin, which is all mucosal in fish, the myeloid CD8\(^+\) MHC II\(^+\) subpopulation, residing in the epidermis, transcribes DC marker genes, such as CD11b, DC-SIGN, CD80/86, CD83, LAMP3, or CD80/86, but no T cell markers. Moreover, the expression of both MHC class I–associated molecules (tapasin, calreticulin, and ERP57) and MHC class II–associated molecules (cathepsin Z and CD80/86) confirms that these cells contain the machinery needed for professional Ag presentation (53). Supporting our phenotypic characterization, trout skin CD8\(^+\) dendritic-like cells expressed high CCR7 levels in steady-state conditions. It is well known that DCs reprogram their expression of chemokine receptors during maturation; CCR7 is one of the chemokine receptors expressed on mature DCs that is absent in immature DCs (57). In mammals, upon stimulation, CCR7 guides skin DCs to enter the skin-draining lymph nodes and encounter lymphocytes that are also recruited to the lymph nodes by CCR7 ligands (58). Because fish lack lymph nodes, CCR7 may be maintaining DCs in the mucosa, which is the location in fish where innate and adaptive immune responses are initially orchestrated. Interestingly, in mammals, CCR7 deficiency reduces the numbers of DCs in the peripheral lymph nodes, but this reduction does not affect all DC subpopulations equally; although CD4\(^+\) DC numbers were minimally affected, CD4\(^+\) and CD8\(^+\) DCs were strongly reduced by the lack of CCR7 (50). Further evidence demonstrated that CCR7 is also responsible for maturation of trout skin CD8\(^+\) MHC-II\(^+\) myeloid cells, because only mature human DCs show T cell–activating properties in an auto-MLR (54). A further transcriptional analysis confirmed that trout skin CD8\(^+\) MHC II\(^+\) myeloid cells were in a rather mature state, expressing high CCR7, CXC4, IFN-\(\gamma\), BAFF, LAMP-3, and CD40 mRNA levels, along with intermediate levels of CD80/86 and CD83; this last molecule is only expressed upon DC maturation in mammals (55). Surprisingly, no IL-12 mRNA was detected in these cells, even after stimulation (data not shown). Although cross-presentation in mice takes place in a Th1 environment with IL-12 production, as seen in mice CD8\(^+\) DCs (56) and human CD141\(^+\) DCs (25), human CD141\(^+\) skin DCs do not produce IL-12 either (29). This suggests that the potential of cross-presenting DCs to produce IL-12 is influenced by the tissue environment.

In concordance with this semiautotranscriptional profile, trout skin CD8\(^+\) dendritic-like cells expressed high CCR7 levels in steady-state conditions. It is well known that DCs reprogram their expression of chemokine receptors during maturation; CCR7 is one of the chemokine receptors expressed on mature DCs that is absent in immature DCs (57). In mammals, upon stimulation, CCR7 guides skin DCs to enter the skin-draining lymph nodes and encounter lymphocytes that are also recruited to the lymph nodes by CCR7 ligands (58). Because fish lack lymph nodes, CCR7 may be maintaining DCs in the mucosa, which is the location in fish where innate and adaptive immune responses are initially orchestrated. Interestingly, in mammals, CCR7 deficiency reduces the numbers of DCs in the peripheral lymph nodes, but this reduction does not affect all DC subpopulations equally; although CD4\(^+\) CD8\(^+\) DC numbers were minimally affected, CD4\(^+\) and CD8\(^+\) DCs were strongly reduced by the lack of CCR7 (50). Further evidence demonstrated that CCR7 is also responsible for...
a continuous turnover of DCs from the skin to the draining lymph nodes in a process called steady-state migration; this is corroborated by the fact that some skin DCs in the epidermis already express CCR7 (50). In fact, these skin DCs found in the draining lymph nodes, without having received inflammatory signals, are in a semimature state, showing high MHC class II levels and intermediate expression of costimulatory molecules, such as CD80 or CD86 (50). This characteristic DC expression profile has been cataloged as a “semimature” state and is believed to induce tolerance (59), because DC maturity or activation levels at the time of Ag encounter determine the promotion of tolerance instead of immunity (1, 8). Trout skin CD8+ dendritic-like cells also showed high CCR7 and MHC class II expression levels and had levels of costimulatory molecules that could be upregulated by virus infection, zymosan, or poly I:C, suggesting that they are also in this semimature state that regulates peripheral tolerance. Because fish lack lymph nodes, the identification of semimature CD8+ DCs in trout skin suggests that, in teleosts, both tolerance and presentation of foreign Ags crossing epithelial barriers are immediately triggered in the skin. We show in this study that the trout skin CD8+ DC-like subset induced the regulation of FoxP3 in both isogeneic and allogeneic splenocytes in the absence of additional stimuli. In contrast, skin CD8+ DC-like cells previously stimulated with TNP–KLH induced a cytotoxic T cell profile in isogeneic splenocytes, as concluded from upregulated levels of T-bet and Eomes. These observations suggest the potential of skin CD8+ DC-like cells to induce tolerance and immunity in rainbow trout. In agreement with this hypothesis, previous studies showed that fish mucosal surfaces, including the skin, are rich in CD3+ T cells (39), suggesting that the establishment of tolerance or activation of T cells in response to water-borne Ags is a local process. Consequently, higher percentages of myeloid CD8α+MHC II+ cells were identified in other mucosal tissues, such as gills or gut, in comparison with the lower percentages observed in lymphoid tissues, such as the spleen or head kidney. Further characterization of these cells in other tissues remains to be done; we focused on skin DCs in this study because the percentage of CD8α+MHC II+ cells was higher in the skin, and because it was in the skin where human DCs with functional homology to the CD8α DC lineage had been fully characterized (29).
Unexpectedly, spleen CD8α+MHC II+ cells of the myeloid gate transcribed several T cell markers, such as CD3 and TCRα, along with the transcription of DC marker genes. Skin CD8α+MHC II+ cells from the lymphoid gate also transcribed CD3 but did not contain any TCR transcripts. Because we are lacking specific Abs against trout surface CD3 or TCRα, we cannot unequivocally state whether these two populations correspond to a heterogeneous cell population or whether they correspond to a true DC subpopulation. In fact, enriched rat CD45+CD2+ cells also transcribed several T cell markers, such as CD3 and TCRγδ, along with the transcription of BAFF, IFN-γ, MHC class I, MHC class II, CD40, CD83, CD-SIGN, tapasin, and calreticulin after water-borne infection with VHSV, an RNA virus. Moreover, the transcriptional profiles observed in the MLRs performed in this study are consistent with a capacity of skin CD8α+ DCs to activate CD8α+ T cells, because a significant augmentation of T-bet and Eomes expression was observed when splenocytes were primed with purified isogenic CD8α+ DCs in mice, whereas tissue cross-presenting DCs are defined by surface expression of MHC class II and CD8, along with the transcription of DC marker genes. Skin CD8α+MHC II+ cells from the lymphoid gate also transcribed CD3 but did not contain any TCR transcripts. Because we are lacking specific Abs against trout surface CD3 or TCRα, we cannot unequivocally state whether these two populations correspond to a heterogeneous cell population or whether they correspond to a true DC subpopulation. In fact, enriched rat CD45+CD2+ cells also transcribed several T cell markers, such as CD3 and TCRγδ, along with the transcription of BAFF, IFN-γ, MHC class I, MHC class II, CD40, CD83, CD-SIGN, tapasin, and calreticulin after water-borne infection with VHSV, an RNA virus. Moreover, the transcriptional profiles observed in the MLRs performed in this study are consistent with a capacity of skin CD8α+ DCs to activate CD8α+ T cells, because a significant augmentation of T-bet and Eomes expression was observed when splenocytes were primed with purified isogenic CD8α+ DCs stimulated earlier with TNP–KLH. Finally, the fact that these cells coexpressed distinctive mammalian markers of different cross-presenting DC subsets strongly supports the ascription of cross-presenting cells in vertebrates to a common DC lineage that arose ≥450 million years ago with the appearance of adaptive immunity.

**Acknowledgments**

We thank Sergio Ciordia (Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas) for technical expertise in proteomic analysis, Ana Vicente Montaña (Centro Nacional de Microscopía Electrónica-Universidad Complutense de Madrid) for technical support with scanning electron microscopy, Dr. Juan Jose Muñoz Oliveira (Centro de Citometría y Microscopía de Fluorescencia-Universidad Complutense de Madrid) for technical assistance with confocal microscopy, and Dr. M. Ototake and Dr. J. Kurita (Fisheries Research Agency, Japan) for providing the clonal fish. The technical support of Lucía González and Susann Schares is also greatly appreciated.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


**TELEOST SKIN CD8α+ DENDRITIC-LIKE CELLS**


**FIGURE S1.** Characterization of anti-trout MHC II mAb. Rainbow trout head kidney protein lysates were used to test the specificity of the 1A1-5C10 mAb by Western blot (A) and immunoprecipitation assays (B). The 30 KDa protein observed in immunoprecipitation (indicated by an arrow) was excised manually from silver-stained gels and digested. (C) Digested peptides were subjected to analysis with Triple TOF 5600 mass spectrometer. MS and MS/MS data were acquired and searched against the NCBI database using the Mascot Server v. 2.5. The protein was identified as *Oncorhynchus mykiss* MHC class II beta chain (accession number: gi|5823078 Protein). The individual peptides identified are also annotated, including sequence, ion score (peptide score with Mascot search engine), m/z (experimental mass-to-charge ratio), mr (relative molecular mass calculated from sequence), charge and e-value (expectation value for the peptide match).
FIGURE S2. Real-time PCR primers used in this study. Accession numbers (Genbank), forward and reverse primer sequences and PCR product sizes (in base pairs) are indicated.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genbank</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAFF</td>
<td>DQ218467</td>
<td>ATGTTTGATGCTTATTCTCTGGCAGGT</td>
<td>TGGGACTCTGTTACTGTGTTGTGA</td>
<td>98</td>
</tr>
<tr>
<td>Batf3</td>
<td>CA345618</td>
<td>CACAGAGACGATGAGTTGGA</td>
<td>TTGGCTCTTCAGACAGAAGACTGCTACCC</td>
<td>95</td>
</tr>
<tr>
<td>Calreticulin</td>
<td>NM_001124478</td>
<td>CAGCTGACACCTCCACCCACACAGAAT</td>
<td>CTCTTCTACATCACTCAACACCATGG</td>
<td>116</td>
</tr>
<tr>
<td>Cathepsin Z</td>
<td>BT073433</td>
<td>CATCCAGAACTACACTCTCTGTTGGA</td>
<td>CTGGTGAAGCGCTCCATTTCTCTG</td>
<td>137</td>
</tr>
<tr>
<td>CCR7</td>
<td>JX982103</td>
<td>TCTACTGATTACCCCACACAGACAAT</td>
<td>AAGGATTAGGCAAGATTGAATGCCGTT</td>
<td>125</td>
</tr>
<tr>
<td>CD3</td>
<td>NM_001165113</td>
<td>CCTGATTGGAGTAGCTGTCTAC</td>
<td>GCTGTACCTGAGATCTGCTCCAT</td>
<td>175</td>
</tr>
<tr>
<td>CD8α</td>
<td>NM_001124263</td>
<td>AGTCTGACAAAGGGGAGGAGG</td>
<td>GTGGTCAATGGCATACAGTGTG</td>
<td>123</td>
</tr>
<tr>
<td>CD8β</td>
<td>NM_001124008</td>
<td>GGGTGCAAGCTCAAGGAGGAAAAGGCAATG</td>
<td>GCTTGACAACTGCTCCACTTTCT</td>
<td>105</td>
</tr>
<tr>
<td>CD11b</td>
<td>AM713180</td>
<td>TTACATTTGTGCTGTCAAGAGG</td>
<td>AAGAACACAGGCAAGACAGCCAGACTCGTCA</td>
<td>104</td>
</tr>
<tr>
<td>CD40</td>
<td>QG169787</td>
<td>TGGACTTGAATCTTAAAGGGGAC</td>
<td>GATGGCTTCACATCCAGGAGGATTAG</td>
<td>124</td>
</tr>
<tr>
<td>CD40L</td>
<td>EF160131</td>
<td>GAGTGGTCAATGTTTCCATCTTACCT</td>
<td>GCTTGACAGCTTTCTTTCAACT</td>
<td>106</td>
</tr>
<tr>
<td>CD80/86</td>
<td>NM_001124414</td>
<td>GGTGTTTCTGTGTTCTGTTCTACT</td>
<td>AACTTGCTGCTCCCTCTCTCTC</td>
<td>109</td>
</tr>
<tr>
<td>CD83</td>
<td>AY263797</td>
<td>GCTGGTGAAGCAGCAGCTGTG</td>
<td>TGGGACTCTAAGGCAAGACTCTG</td>
<td>105</td>
</tr>
<tr>
<td>CD103</td>
<td>CDQ67442</td>
<td>CATCCAGAACTACACTCTCTGTTGGA</td>
<td>CTCTTCTACATCACTCAACACCATGG</td>
<td>137</td>
</tr>
<tr>
<td>CD141</td>
<td>KP203844</td>
<td>CAGAATCTGACAGCTTGCAAGACAGAAAGAAAA</td>
<td>ACTTTCTGCTGCAAGACAGCTGTCTCTG</td>
<td>103</td>
</tr>
<tr>
<td>CXCR4</td>
<td>NM_001124342</td>
<td>GTGACTGTTGATCTACACTGCT</td>
<td>GAGGCTTGCGAAGACAGACAGCTG</td>
<td>199</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>NM_001124633</td>
<td>GAGAAGGAAAGGGGATTGGAG</td>
<td>CCCATGTCATTCTCTGACT</td>
<td>108</td>
</tr>
<tr>
<td>EFlα</td>
<td>AF498320</td>
<td>GATCCAGAAGGAGGCTGACCA</td>
<td>TTAGGTGGACCTTTCTCCAC</td>
<td>170</td>
</tr>
<tr>
<td>Eomes</td>
<td>JF719912</td>
<td>AACAATGTTATTGGATGCTGTTG</td>
<td>CATCTTTGTACCTTGATGTTTG</td>
<td>110</td>
</tr>
<tr>
<td>ERp57</td>
<td>NM_001281398</td>
<td>GCCTACTGACAGCTTGACAGAG</td>
<td>GATGGCTTCACATCCAGGAGGATTAG</td>
<td>124</td>
</tr>
<tr>
<td>FoxP3</td>
<td>FM83710</td>
<td>CCAGACACGAGGTTGAGG</td>
<td>TGGGACAGCGGTCTTCCTCA</td>
<td>294</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>NM_001124620</td>
<td>GAAGGGCTTGGTCCGAGGTCA</td>
<td>TGTGATTTGACGACTTCGTGG</td>
<td>119</td>
</tr>
<tr>
<td>IRF-4</td>
<td>AJ829677</td>
<td>CATGGGCCACCTTTCTGACTCC</td>
<td>GCATACCCCTGACAGTCTGAGA</td>
<td>432</td>
</tr>
<tr>
<td>IRF-8</td>
<td>AJ829674</td>
<td>CCGAGGAGGACGAGAGAGAAGTAGAAGAAAG</td>
<td>GCGGACTTGAAGGAACAGAAGCCCAT</td>
<td>257</td>
</tr>
<tr>
<td>LAMP-3</td>
<td>HE608240</td>
<td>CATGAAAAGCTGTTCCACACTGCTC</td>
<td>GACTCAACCTCTCCTCTCACC</td>
<td>156</td>
</tr>
<tr>
<td>MHC-I</td>
<td>AF115522</td>
<td>GACAGTCGGTCCCTCAAGGTG</td>
<td>TGAGAAGGGTCCCCATCAGTGTG</td>
<td>175</td>
</tr>
<tr>
<td>MHC-II</td>
<td>AF296384</td>
<td>ACACCTTTATCTGCACAGT</td>
<td>TCTGGGTTGGAAGCTAGACT</td>
<td>160</td>
</tr>
<tr>
<td>Perforin</td>
<td>AM295251</td>
<td>GGAAGCAGACCCGTTGAGTTGA</td>
<td>TCTAGGGAGGGGGTACATAG</td>
<td>162</td>
</tr>
<tr>
<td>T-bet</td>
<td>NM_001195793</td>
<td>GGTTCCTCACTGCTCTTACATGTAG</td>
<td>CTATGAAATGGTTGCTCTTTCCAGGCAGGG</td>
<td>127</td>
</tr>
<tr>
<td>Tapasin</td>
<td>AAZ66042</td>
<td>TCGTCCATAAACCACAGTGTTGAGA</td>
<td>CAGTGCGTAATGCTAGTGAAGGACAGGAGAGAG</td>
<td>129</td>
</tr>
<tr>
<td>TCRα</td>
<td>U50991</td>
<td>ACGACCTTGGAATTTATTCACAAGA</td>
<td>GCTCTCACATTTCTCTGACACCTA</td>
<td>122</td>
</tr>
<tr>
<td>TCRγ</td>
<td>EU072700</td>
<td>GAGAAGAACACCCGTTGAGTTGA</td>
<td>GACATGGTGGTGAGGTATCTCTTGT</td>
<td>134</td>
</tr>
<tr>
<td>TLR-1</td>
<td>NM_001166101</td>
<td>CACAGGCCCTGGTTGATGTTCT</td>
<td>CTCTTCAGAATGGTCCGACACCCAGG</td>
<td>90</td>
</tr>
<tr>
<td>TLR-2</td>
<td>HE979560</td>
<td>GATCCAGACGAAACACCTCTCAACAT</td>
<td>CTCCAGACATGAAAGTTGACAAACGGAT</td>
<td>129</td>
</tr>
<tr>
<td>TLR-3</td>
<td>DQ459470</td>
<td>AGGCTTTTGTCTGCTCAGAG</td>
<td>GCTCTACTGATTCTTGGTGGAGAGCCG</td>
<td>61</td>
</tr>
<tr>
<td>TLR-5</td>
<td>NM_001124208</td>
<td>TTGACTTATCTCCACAGGATGCA</td>
<td>CTCTTTAATGTTGACAAACCACAAATG</td>
<td>135</td>
</tr>
<tr>
<td>TLR-7</td>
<td>GQ422119</td>
<td>TACAGCTGTTGAAATGACTCTCCTC</td>
<td>CAACATTCTGACATTTGCGTCTGTA</td>
<td>89</td>
</tr>
<tr>
<td>TLR-8a2</td>
<td>GQ422120</td>
<td>CATCTATGCTCTCCTACACGAGACCC</td>
<td>GTGCTCCCAATATGACAAACCTTCTT</td>
<td>146</td>
</tr>
<tr>
<td>TLR-9</td>
<td>NM_001129991</td>
<td>TCTTACATAGCTGAAAGGGGCCTCA</td>
<td>GTTCCACATGGAGAAGATGGTTTT</td>
<td>137</td>
</tr>
<tr>
<td>TLR-22</td>
<td>NM_001124412</td>
<td>TGGACAATGACGCTCTTCTACC</td>
<td>GAGCTGATGGTTGCAATGAGG</td>
<td>151</td>
</tr>
</tbody>
</table>
Fig. S3

**FIGURE S3.** Comparison of skin CD8+MHC II+ cells from the lymphoid gate and the CD8+ DC-like subpopulation. Trout leukocytes from spleen (A) and skin (B) were labelled with mAbs against trout CD8α and MHC II β chain. Leukocytes were gated as lymphoid (L) and myeloid (M) cells, on the basis of FSC and SSC (shown on the left dot plots). Then, CD8 and MHC II fluorescence intensity was analyzed on both lymphoid and myeloid gates. For experimental purposes, skin CD8+MHC II+ events from the myeloid gate were isolated, as well as splenic CD8+MHC II+ events from the myeloid gate and skin CD8+ MHC II+ events from the lymphoid gate. (C) Comparison of gene expression profile of skin CD8+MHC II+ cells from the lymphoid gate and the CD8+ DC-like subpopulation. Skin CD8+MHC II+ cells from the lymphoid gate and skin CD8+ dendritic-like cells (myeloid gate) were isolated by cell sorting and RNA was obtained. The relative expression of the indicated genes to the endogenous control EF-1α was calculated for each sample, and then mean values from three independent experiments, containing three animals per experiment, were calculated. Results are shown as mean ± SD.
FIGURE S4. Comparison of gene expression pattern of splenic CD8+ T cells, macrophages and skin CD8+ dendritic-like cells. Trout leukocytes from spleen and skin were purified. Skin CD8+ dendritic-like cells (myeloid gate) were isolated by cell sorting and RNA was obtained. In parallel, splenic CD8+ T cells (CD8+MHC II- events from the lymphoid gate) from the same individuals were isolated by cell sorting and RNA was obtained. RNA was also extracted from the RTS11 trout macrophage cell line following the same procedure. (A) Analysis of the gene expression pattern of T cell and antigen-presenting cell (APC) markers on CD8+ DCs, CD8+ T cells and macrophages. (B) Analysis of the gene expression pattern of MHC class I and class II-associated genes on CD8+ DCs and macrophages. In each case, the relative expression of the indicated genes to the endogenous control EF-1α was calculated for each sample, and then mean values from three independent experiments, involving three animals per experiment, were calculated. Results are shown as mean ± SD.