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Head Kidney-Derived Macrophages of Common Carp (Cyprinus carpio L.) Show Plasticity and Functional Polarization upon Differential Stimulation

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Cells from the myeloid lineage are pluripotent. To investigate the potential of myeloid cell polarization in a primitive vertebrate species, we phenotypically and functionally characterized myeloid cells of common carp (Cyprinus carpio L.) during culture. Flow cytometric analysis, Ab labeling of cell surface markers, and light microscopy showed the presence of a major population of heterogeneous macrophages after culture. These head kidney-derived macrophages can be considered the fish equivalent of bone marrow-derived macrophages and show the ability to phagocyte, produce radicals, and polarize into innate activated or alternatively activated macrophages. Macrophage polarization was based on differential activity of inducible NO synthase and arginase for innate and alternative activation, respectively. Correspondingly, gene expression profiling after stimulation with LPS or cAMP showed differential expression for most of the immune genes presently described for carp. The recently described novel Ig-like transcript 1 (NILT1) and the CXCR1 and CXCR2 chemokine receptors were up-regulated after stimulation with cAMP, an inducer of alternative activation in carp macrophages. Up-regulation of NILT1 was also seen during the later phase of a Trypanosoma carassii infection, where macrophages are primarily alternatively activated. However, NILT1 could not be up-regulated during a Trypanoplasma borrelii infection, a model for innate activation. Our data suggest that NILT1, CXCR1, and CXCR2 could be considered markers for alternatively activated macrophages in fish. The Journal of Immunology, 2006, 177: 61–69.

Macrophages play a role in both the innate and the adaptive immune system. In the innate immune system, they act as phagocytic cells, phagocytosing pathogens and producing oxygen and nitrogen radicals. In the adaptive immune system, macrophages act as professional APCs. As such, macrophages can provide a bridge between the innate and adaptive immune response. Altogether, macrophage differentiation exhibits a wide array of functional and phenotypic heterogeneity (1). Within this functional heterogeneity, classically and alternatively activated macrophages (caMF4 and aaMF, respectively) are proposed to represent the extremes of a continuum (2–4). In literature, a further distinction has been made between classical and innate activation of macrophages (2). Classical activation is induced by stimulation with both IFN-γ and a microbial trigger such as LPS, whereas innate activation is induced by stimulation with LPS (or other microbial triggers) alone (2). Although classically and innate activated macrophages are induced by different stimuli, their functions overlap. Innate activated macrophages have microbialidal activity and produce proinflammatory cytokines, reactive oxygen species (ROS) and NO (5). caMF find their role in type I immune responses against intracellular pathogens by the production of ROS and NO. aaMF are active in type II immune responses against extracellular pathogens by showing increased phagocytic activity and enhanced gene expression of MHC class II. Furthermore, aaMF increase their production of factors involved in tissue remodeling and repair and are able to inhibit type I inflammations (6). Macrophage activation in fish has been well studied with regard to NO production (7–11) and ROS (12–15). Recently, we described carp arginase gene expression and activity, which can be used as markers for alternatively activated macrophages, and we proposed an evolutionary conservation of alternatively activated macrophages down to teleost fish (16).

The aim of the present study was to investigate the macrophage polarization in teleosts, using functional assays and gene expression profiling. Hence, we developed a primary cell culture system of carp head kidney-derived macrophages. In fish, only a few such culture models exist for goldfish, rainbow trout, and brook trout (17–19). We studied morphological changes by flow cytometry and light microscopy. We investigated functional changes by determining phagocytic ability, the ability to produce radicals (both ROS and NO), and by measuring arginase activity. A well-accepted way to study polarization is by determining gene expression profiles after differential stimulation. We quantified gene expression, by real-time quantitative PCR, in head kidney-derived macrophages stimulated with LPS or cAMP. Gene expression of carp CXCR1 and CXCR2 was up-regulated after cAMP stimulation.

In addition to the CXCR1 and 2, head kidney-derived macrophages expressed the recently identified novel Ig-like transcripts (NILT)-1 and NILT2 (20) at different levels following stimulation. NILT1 and NILT2 are polymorphic receptors belonging to the Ig

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1 This study was supported by a grant from the Dutch Organization for Scientific Research.

2 Current address: Scottish Fish Immunology Research Centre, University of Aberdeen, Tillydrone Avenue, AB24 2TZ Aberdeen, U.K.

3 Address correspondence and reprint requests to Dr. Geert F. Wiegertjes, P.O. Box 338, 6700 AH Wageningen, The Netherlands. E-mail address: geert.wiegertjes@wur.nl

4 Abbreviations used in this paper: caMF, classically activated macrophage; aaMF, alternatively activated macrophage; DEPC, diethyl pyrocarbonate; DHR, dihydrorhodamine; FSC-H, forward scatter; HKL, head kidney leukocyte; iNOS, inducible NO synthase; NILT, novel Ig-like transcript; PI, propidium iodide; ROS, reactive oxygen species; SSC-H, side scatter; TREM, triggering receptors expressed on myeloid cell.
superfamily, which, in general, recognize pathogen-associated molecular patterns. These type of receptors frequently exist in pairs with antagonistic signaling functions, are coexpressed on the same cell, and bind similar, if not identical, ligands (21). The cytoplasmic region of NILT1 contains a cytoplasmic ITAM and of NILT2 an ITIM. The use of NILT1, CXXCR1, and CXXCR2 as possible surface markers for alternatively activated macrophages in fish is discussed.

Materials and Methods

Animals

Common carp (Cyprinus carpio L.) were reared in the central fish facility “De Haar-Vissen” at 23°C in recirculating UV-treated tap water and fed pelleted dry food (Trouvit) daily. R3 heterozygous carp are the offspring of a cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain) (22). Carp were between 9 and 11 mo old. All studies were performed with approval from the animal experimental committee of Wageningen University.

Cell culture for macrophages

Carp head kidney leukocytes (HKL) were cultured essentially as described before for goldfish and trout (17–19). Briefly, fish were euthanized with 0.25 g/l tricaine methane sulfonate (Crescent Research Chemicals), buffered with 0.38 g/l NaHCO₃, and bled by syringing from the caudal vein, and head kidneys were removed aseptically. Head kidneys were gently passed through a 100-µm sterile nylon mesh and rinsed with homogenization buffer (incomplete-NMGGF15 medium containing 50 U/ml penicillin, 50 µg/ml streptomycin, and 20 U/ml heparin (Leo Pharma)) (17). Cell suspensions were layered on 51% (1.071 g/cm³) Percoll (Amersham Biosciences) and centrifuged at 450 × g for 25 min at 4°C without brakes. Cells at the medium/Percoll interface were removed and washed twice. Cell cultures were initiated by seeding 1.75 × 10⁶ HKL in a 75-cm² culture flask containing 20 ml of complete-NMGGF15 medium (incomplete-NMGGF15 medium supplemented with 5% heat-inactivated pooled carp serum and 10% bovine calf serum (Invitrogen Life Technologies)) with 50 U/ml of penicillin and 50 µg/ml streptomycin. Cells were incubated at 27°C, and head kidney-derived macrophages were harvested after 6 days by placing the flasks on ice for 10 min and gentle scraping.

Flow cytometry

Flow cytometry was used to follow the development of the cells, and forward scatter (FSC-H, reflecting cell size) and side scatter characteristics (SSC-H, reflecting internal cell complexity) were recorded for 10⁶ events per sample, using a flow cytometer (Beckman Coulter). For all cytometric measurements, the same settings were used: FS 350 V, gain 2; SS 700 V, gain 10; FL-1 800 V, gain 1; FL-2 670 V, gain 1; FL-3 675 V, gain 1 and FL-4 950 V, gain 1. The baseline offset was on and the discriminator voltage was set at FS at 20. For the phagocytosis assay, a gate was set to measure cells only and not the (smaller) bacteria. Propidium iodide (PI; 0.1 µg/ml) was added to each sample to detect and gate out PI⁺ cells, and 100 µl of a standard diluted bead solution (Fluoresbrite YG Carboxylate Microspheres (10 µm); Polysciences) was added to determine the amount of cells in culture. Cell populations were identified by FSC-H/SSC-H as described by van Kempen et al. (23) for freshly isolated HKL and by MacKenzie et al. (19) for populations were identified by FSC-H/SSC-H as described by van Kempen et al. (23). Subsequently, cells were incubated with a 1/50 dilution of a secondary Ab (rabbit-anti-mouse RPE PE) and goat-anti-mouse FITC (DakoCytona) for 30 min on ice, washed, and resuspended in 200 µl flow cytometer medium containing PI (0.1 µg/ml) to detect and gate out PI⁺ cells. Per sample 10⁶ events were measured by flow cytometer. A control sample incubated with the secondary Ab only, was included in each experiment and consistently found to be negative.

Light microscopy

For light microscopy, cell suspensions of freshly isolated HKL and head kidney-derived macrophages were pelleted (10 min, 450 × g). Cell pellets were resuspended in 1% (w/v) K₂CO₃, 2% (v/v) glutaeraldehyde, and 1% (w/v) OsO₄ in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h at 0°C and subsequently washed in double-distilled water, dehydrated in alcohol and propylene oxide, and embedded in Epon 812 (Electron Microscopy Science). Semithin sections were cut on a Reichert Ultracut E (Leica), stained with 1% (w/v) toluidine blue O, 1% (w/v) borax on a hot plate for 1 min, rinsed with tap water, and embedded in depex (Serva).

Phagocytosis, radical production, arginase activity and nitrite production

Freshly isolated HKL or head kidney-derived macrophages (5 × 10⁶) were seeded in 100 µl of rich-NMGGF15 medium (incomplete-NMGGF15 medium supplemented with 2.5% heat-inactivated pooled carp serum and 5% bovine calf serum (Invitrogen Life Technologies)) in wells of a 96-well flat-bottom culture plate.

For measurement of phagocytosis, cells were stimulated with LPS (50 µg/ml, Escherichia coli, L2880; Sigma-Aldrich) or left untreated to measure basal phagocytosis, and incubated for 18 h at 27°C. Stimulated cells were resuspended by pipetting, transferred to flow cytometry tubes, and incubated with FITC-stained Staphylococcus aureus (5 × 10⁹) for an additional hour at 27°C. Bacteria (Staphylococcus aureus) (Pansorbin; Merck Biosciences) were stained by overnight incubation with FITC (5 µg/ml) at room temperature and continuous rotation. Bacteria were washed, at least three times, with incomplete medium to remove all unbound FITC. Phagocytosis was stopped by placing the tubes on ice and adding 1–2 ml of ice-cold PBS. PI (0.1 µg/ml) was added to each sample to detect and gate out PI⁺ cells, and fluorescence of nonphagocytosed bacteria was quenched by adding 10% (v/v) urea blue (130 µg/ml). A total number of 10⁵ events in the cell gate was measured by flow cytometry, the cell gate excluded the free bacteria from the measurements.

For measurement of radical production, cells were stimulated with PMA (0.1 µg/ml) or left untreated as controls. At the same time, dihydroxyamine (DHR) (23) (10 µg/ml, D164; Sigma-Aldrich, 28.8 µM) was added to all samples, and samples were incubated for 1 h at 27°C. Cells were resuspended by pipetting and transferred to flow cytometry tubes, PI (0.1 µg/ml) was added to each sample to detect and gate out PI⁺ cells, and 10⁶ events were measured by flow cytometer.

For measurement of arginase activity, cells were stimulated with the cAMP analog dibutyryl cAMP (0.5 mg/ml, dibutyryl cyclic adenosine monophosphate, D6572; Sigma-Aldrich), or left untreated, and incubated for 18 h at 27°C. Arginase activity was measured essentially as described by Green et al. (28): to 75 µl of 0.1% (w/v) N-acetyl-L-lysine at pH 7.5 was added, and the mixture was incubated for 10 min at 37°C. Reaction was stopped by adding 400 µl of acid mixture containing H₂SO₄, H₂O₂, and H₂O (1:3:7), then to each reaction 25 µl of 9% e-isonitrososperiphenone (in 100% ethanol) was added and incubated for 45 min at 100°C. After 10 min cooling in the dark, the absorbance was read at 540 nm, and arginase activity (mU per million cells, mU) was calculated by comparison with a urea standard curve.

For measurement of nitrite production, cells were stimulated with LPS (50 µg/ml), or left untreated, and incubated for 18 h at 27°C. Nitrite production was measured essentially as described by Green et al. (28): to 75 µl of cell culture supernatant, 100 µl of 1% (w/v) sulfanilamide in 2.5% (v/v) phosphoric acid and 100 µl of 0.1% (w/v) N-naphthyl-ethylendiamine in 2.5% (v/v) phosphoric acid were added in a 96-well flat-bottom plate. Absorbance was read at 540 nm (with 690 nm as a reference), and nitrite concentrations (µM) were calculated by comparison with a sodi um nitrate standard curve.

Gene expression profiling of LPS and cAMP stimulated head kidney-derived macrophages

To analyze the functional heterogeneity of head kidney-derived macrophages, gene expression levels of an array of genes (TNF-α, IL-11, IL-1β, IL-1βR, IL-10, IL-12P35, IL-12P40, IL-12P40.2, IL-12P40.3, iNOS, arginase 1, arginase 2, CXCa, CXCb, CXCR1, CXCR2, MHC-II, NILT1, and...
NILT2) were measured after LPS or cAMP stimulation. Head kidney-derived macrophages were stimulated with LPS (50 μg/ml) or CAMP (0.5 mg/ml) or left untreated as control and incubated for 6 h at 27°C. To study NILT gene expression in more detail, samples were taken after 0, 3, 6, 9, and 18 h of incubation at 27°C. From all samples, RNA was isolated, cDNA synthesized, and gene expression levels were determined by means of real-time quantitative PCR (see later sections).

Gene expression during Trypanosoma carassii and Trypanoplasma borrelii infections

T. carassii was cloned and characterized by Overath et al. (29). T. borrelii was cloned and characterized by Steinhagen et al. (30). Parasites were maintained by syringe passage through carp. Thirty-two carp were i.p. injected with 10,000 T. carassii per fish and 5 were left untreated as control. At every time point, 20 carp were i.p. injected with 10,000 T. borrelii per fish and 5 were left untreated as control. At every time point, four fish for the T. carassii and five fish for the T. borrelii experiment were sacrificed and their head kidneys removed. Head kidneys were snap frozen in liquid nitrogen and stored at −80°C. From all head kidneys, RNA was isolated, cDNA synthesized, and gene expression levels were determined by means of real-time quantitative PCR (see later sections).

RNA isolation, DNase treatment, and first strand cDNA synthesis

RNA was isolated from a 27-mm3 head kidney (organ) or 2 × 105–5 × 106 cells per treatment using the RNaseasy Mini kit (Qiagen), including the accompanying DNase I treatment on the columns, according to the manufacturer’s protocol. End elution was performed with 25 μl of diethyl pyrocarbonate (DEPC)-treated water. RNA concentrations were measured by spectrophotometry (Genequant; Pharmacia Biotech), and 1 μl was analyzed on a 1% agarose gel to check the integrity. Routinely, 10 μg of RNA approximately was isolated from 27 mm3 head kidney (organ) and 15 μg of RNA from 2 to 5 × 106 cells. RNA was stored at −80°C until further use. For each cDNA synthesis, a negative sample (non-RT), to which no reverse transcriptase was added, and a positive sample containing the reverse transcriptase were included. After DNase treatment, 1 μg of total RNA was combined with random primers (300 ng; Invitrogen Life Technologies), 1 μl of Superscript RNase H Reverse Transcriptase II (200 U/μl; Invitrogen Life Technologies), 1 μl of DTT (0.1 M), and 1 μl of 106 cells. RNA was stored at −80°C until further use. For each cDNA synthesis, a negative sample (non-RT), to which no reverse transcriptase was added, and a positive sample containing the reverse transcriptase were included. After DNase treatment, 1 μg of total RNA was combined with random primers (300 ng; Invitrogen Life Technologies), 1 μl of Superscript RNase H Reverse Transcriptase II (200 U/μl; Invitrogen Life Technologies) was added. To each negative sample, 1 μl of DEPC-treated water was added. All samples were incubated at 37°C for 50 min. Reactions were stopped by adding MilliQ water up to 100 μl and cDNA stored at −20°C until use.

Real-time quantitative PCR

Specific real-time quantitative PCR primers (Table I) were designed with the Primer Express software (Applied Biosystems). To 5 μl of 10 times-diluted cDNA, 7 μl of Sybr Green Master Mix (Stratagene), forward and reverse primer (300 nM each), and MilliQ water up to 14 μl were added. Quantitative PCR was performed in a 72-well Rotor-Gene centrifugal real-time thermal cycler (Rotor-Gene 2000 Corbett Research). The following cycling conditions were used: one holding step of 10 min at 95°C, followed by 40 cycles of 20 s at 94°C, 30 s at 60°C, and 30 s at 72°C; an incubation for 1 min at 60°C was followed by a melting curve from 60 to 90°C in steps of 1°C with 5 s waiting. At the end of each cycle and during the waiting steps in the melting curve, fluorescence intensities were measured. Raw data were analyzed using the comparative quantitation of the Rotor-Gene Analysis Software version 5.0. Data were further analyzed using the Pfaffl method (31), and average efficiencies per run per gene were used. Gene expression of 40S in each sample was highly constant and used to normalize the data. Specificity of the amplification was ensured by checking the melting temperature and profile of each melting curve. The product of each template was checked at least once by sequencing.

Statistics

Phagocytosis, radical production, arginase activity, nitrite production, and gene expression differences between the differential stimulated head kidney-derived macrophages and during the infections were tested for significance by Student’s t test. A value of p < 0.05 was accepted as significant. NILT1 and NILT2 gene expression in the head kidney-derived macrophages over time were analyzed by a repeated measurement model (PROC GLM, SAS, version 8.02; SAS Institute) (32), with time as the repeating factor. The treatments were tested for significance against the interaction of treatment and individual. A value of p < 0.05 was accepted as significant.

Results

Macrophage cell culture: characterization by cell number, surface marker staining, and morphology

Carp HKL were separated on a 51% Percoll layer and cultured in vitro for several days. A pilot experiment where cells were cultured up to 8 days and analyzed by flow cytometer indicated an expanding cell population until day 7. To characterize these in

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<th>Melting Temperature (°C)</th>
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*There are three arginase 2 isoforms known, AJ618955/AJ971265-66; this primer set amplifies all three.*
vitro cell cultures, we followed their development by flow cytometry, determining cell number, size, and internal complexity (FSC-H and SSC-H, respectively) and expression of cell surface markers up to day 6 in culture.

During the first 2 days of culture, total cell numbers decreased drastically, while after 4 days of culture, cell numbers increased again, indicating proliferation (Fig. 1A). At the start of the culture (day 0), three cell populations could be identified based on FSC-H/SSC-H dot plots (Fig. 1B). First, a population of lymphocytes and precursor cells was characterized by a low FSC-H/SSC-H (lower left hand corner). Most of the WCI12⁺ cells (B lymphocytes) could be found in this population. Over time, during the first 2 days of culture especially, this lymphocyte population decreased dramatically (Fig. 1, B and C). Second, a population of monocytes was characterized by a medium FSC-H/SSC-H (spreading out to the middle). A low percentage of WCL15⁺ cells (macrophages, monocytes, and basophilic granulocytes) could be found primarily in this population. Over time, the relative percentage of WCL15⁺ cells increased steadily (Fig. 1C). Third, a population of neutrophilic granulocytes was characterized by a high FSC-H/SSC-H (upper right hand corner). Almost all of these cells were TCL-BE8⁺ (neutrophilic granulocytes, and monocytes). During the first 2 days of culture, the relative percentage of TCL-BE8⁺ cells remained constant (Fig. 1C). However, because the total cell number decreased rapidly (Fig. 1A), the absolute number of TCL-BE8⁺ cells also decreased. From day 4 onward, the percentage of TCL-BE8⁺ cells increased again.

After 4 days, cell cultures consisted of two populations (Fig. 1B). One major population of head kidney-derived macrophages with a high FSC-H/SSC-H were TCL-BE8⁺, WCL15⁺, or TCL-BE8⁺ WCL15⁺, as confirmed by fluorescence microscopy (data not shown). The second population was smaller in number and consisted of precursor cells with a low FSC-H/SSC-H that were negative for all three cell surface markers used. During the last 2 days of the culture especially, the head kidney-derived macrophages further increased in number, both relative and absolute. Changes in cell number were also reflected by the changes in the percentage of PI⁺ cells. The percentage of PI⁺ cells increased from 5% at day 0 to 10% at day 2 but decreased again to 7% at day 6.

Light microscopy (Fig. 2) supported the decrease in lymphocytes and granulocytes and the increase in myeloid cells as detected by the Ab labeling. Within this myeloid cell population, there is still a degree of heterogeneity visible.

**Phagocytic ability, (oxygen) radical production, arginase activity, and nitrite production are high in head kidney-derived macrophages**

Carp HKL were cultured in vitro for 6 days to obtain head kidney-derived macrophages, which were assessed for cell function by measuring phagocytosis and (oxygen) radical production by flow cytometry and arginase activity and nitrite production by a calorimetric method.

The phagocytic ability, the number of cells capable of phagocytosis, was measured both in freshly isolated HKL (day 0) and in head kidney-derived macrophages (day 6). In a pilot experiment, a time curve showed a plateau phase of phagocytosis after 1 h incubation at 27°C, which was chosen as the time of incubation in the following experiments. Head kidney-derived macrophages showed significantly higher basal phagocytic ability (19%) of FITC-labeled bacteria, as compared with freshly isolated HKL (8%). Prestimulation with LPS for 18 h further increased the phagocytic ability both in head kidney-derived macrophages (23%) and in freshly isolated HKL (13%) (Fig. 3A).

Radical production was measured by flow cytometry following incubation with DHR. Head kidney-derived macrophages showed a very high (77%) basal activity, as compared with freshly isolated HKL (22%) (Fig. 3B). While radical production in freshly isolated HKL could be significantly increased by PMA stimulation (51%),
radical production by head kidney-derived macrophages could not be further increased (79%).

Arginase activity was measured after 18 h of incubation with or without cAMP stimulation. Head kidney-derived macrophages showed a significantly higher arginase activity, as compared with freshly isolated HKL (Fig. 3C). This was true both for basal activity (2 vs 6 mU), as well as for cAMP-induced activity (2 vs 14 mU). cAMP stimulation increased the arginase activity of head kidney-derived macrophages significantly.

Nitrite production was measured after 18 h of incubation with or without LPS stimulation. Head kidney-derived macrophages showed a significantly higher ability to produce NO, measured as nitrite in a Griess reaction, as compared with freshly isolated HKL (Fig. 3D). This was true for both basal activity (2 vs 15 μM) and for LPS-induced activity (3 vs 36 μM). LPS stimulation increased the NO production of head kidney-derived macrophages significantly. Basal NO levels were high in head kidney-derived macrophages, considering a stimulation with LPS of 18 h only, already showed a considerable nitrite production of 15 μM.

Gene expression profiles of LPS- or cAMP-stimulated head kidney-derived macrophages are different

RNA was isolated from head kidney-derived macrophages (6-day culture) stimulated for 6 h with LPS or cAMP or from unstimulated cells to assess differences in gene expression as a measure of macrophage polarization. Gene expression levels of an array of immune-relevant genes currently described for carp were studied by real-time quantitative PCR, using 40S and β-actin gene expression as housekeeping gene references. Results with 40S and β-actin were comparable; only the results relative to 40S are shown (Table II).

Gene expression levels of cells stimulated with LPS or cAMP were compared with unstimulated cells, as shown in Table II. In addition, gene expression levels of LPS-stimulated head kidney-derived macrophages were compared with gene expression levels in LPS-stimulated head kidney-derived macrophages from the differentially expressed genes, TNF-α, IL-10, iNOS, CXCα, and NILT2 were significantly higher expressed in LPS-stimulated head kidney-derived macrophages, whereas IL12P40.3, arginase 2, CXCRI, and CXCR2 were significantly higher expressed in cAMP-stimulated head kidney-derived macrophages.

NILT1 gene expression is up-regulated in cAMP-stimulated head kidney-derived macrophages and in T. carassii-infected carp but not in T. borreli-infected carp

From the genes differentially expressed in LPS- or cAMP-stimulated head kidney-derived macrophages, we selected the NILT genes for a more detailed study because of the unknown function of these novel receptors in fish. To further characterize the gene

FIGURE 2. Light microscopic photographs (bar is 10 μm) of freshly isolated HKL (A) and head kidney-derived macrophages (B). Cells were pelleted and fixed, and semithin sections were cut and stained with toluidine blue and borax.

FIGURE 3. Phagocytosis (A), radical production (OH, CO$_3^-$, NO$_3^-$, H$_2$O$_2$, and NO$_2^-$) (B), arginase activity (C), and nitrite production (D) by freshly isolated HKL (day 0) and head kidney-derived macrophages (day 6). Cells (5 × 10$^5$ per 100 μl) were stimulated (●) or left untreated to measure basal activity (□). Cells were stimulated with LPS (50 μg/ml) for phagocytosis (A) and nitrite production (D), with PMA (0.1 μg/ml) for radical production (B) or with cAMP (0.5 mg/ml) for arginase activity (C). Averages and SD of n = 4 fish are given for phagocytosis and radical production. Averages and SD or triplicate measurements of a representative experiment out of n = 7 are given for arginase activity and nitrite production. *, p < 0.05, by Student’s t test, compared with day 0 control. #, p < 0.05 Student’s t test, compared with corresponding time point control.
expression of NILT1 and NILT2, head kidney-derived macrophages were stimulated with LPS, with cAMP, or left untreated as control, and gene expression relative to 40S was followed over time. NILT1 gene expression was significantly up-regulated after stimulation with cAMP. In contrast, LPS did not influence NILT1 expression (Fig. 4A). NILT2 gene expression was consistently up-regulated after LPS stimulation. cAMP stimulation showed a consistent down-regulation of NILT2 (Fig. 4B).

In addition, we measured the in vivo gene expression of both NILT1 and NILT2 in head kidney (whole organ) of carp infected with either *T. carassii* or *T. borreli*. Both NILT1 and NILT2 gene expression were down-regulated during the early phase of infection with *T. carassii* but up-regulated during later time points in the *T. carassii* infection (Fig. 5, A and C). Observed effects were stronger for NILT1 than for NILT2.

The *T. borreli* infection was more severe and lasted shorter than the *T. carassii* infection. During *T. borreli* infection, NILT gene expression was different. NILT1 gene expression was significantly up-regulated only at wk 4 (Fig. 5B), whereas NILT2 gene expression was not significantly regulated (Fig. 5D).

**Discussion**

Our experiments show that carp head kidney-derived macrophages are heterogeneous and, upon in vitro stimulation, can give rise to functionally different polarization states. According to FSC-H/SSC-H profiles, the cell cultures consisted of two populations. A majority of the cells (>80%) with a high FSC-H/SSC-H profile are mature macrophages, as discussed in detail later. The remainder of the cells with a low FSC-H/SSC-H profile most likely are precursor cells of the myeloid lineage, as was shown for goldfish (33). The functional heterogeneity gives rise to macrophage populations that can be activated innate or alternatively. Gene expression profiling suggested that NILT1, CXCR1, and CXCR2 might be used as surface markers for alternatively activated macrophages. The use of NILT1 as putative cell surface marker for aaMF in fish was confirmed in an in vivo experiment.

Until present, carp macrophages have been studied using head kidney-derived leukocytes isolated by Percoll density centrifugation, followed by an adherence step in RPMI 1640 medium with adjusted molarity. This method leads to slightly enriched fractions of macrophages (23). To study macrophage polarization and functional heterogeneity of a primitive vertebrate in more detail, here, we describe a method to culture carp macrophages based on a procedure previously developed for goldfish and trout (17–19). HKL were isolated on a 51% Percoll layer, seeded in NMGFL-15 medium (17), and cultured for 6 days. NMGFL-15 medium enhances myeloid but not lymphoid cell growth of goldfish and trout (17, 18). Indeed, also for carp, the NMGFL-15 medium leads to an early reduction in the number of lymphocytes (WC12− (23), low FSC-H/SSC-H (24)) and supported a subsequent increase in the number of macrophages (WCL5+ TCL-BE8+) (25, 26), high FSC-H/SSC-H (19)). Light microscopy supported head kidney-derived cell cultures to be primarily macrophages with phenotypic heterogeneity.

Head kidney-derived macrophages present at day 6 of culture were functional macrophages as could be seen from their apparent capacity to phagocytose fluorescent bacteria and increased radical production. To measure the ROS production, cells were incubated with DHR. Free radicals and some other molecules (OH, CO3−, "

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**Table II. Gene expression of head kidney-derived macrophages after 6 h stimulation with LPS or cAMP relative to unstimulated cells**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Take-Off Valuea</th>
<th>LPS</th>
<th>cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-αb</td>
<td>21.2</td>
<td>1.14c</td>
<td>0.74</td>
</tr>
<tr>
<td>IL-1β</td>
<td>24.9</td>
<td>6.62c</td>
<td>5.24c</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>22.8</td>
<td>1.33</td>
<td>1.10</td>
</tr>
<tr>
<td>IL-10b</td>
<td>21.4</td>
<td>1.01</td>
<td>0.60</td>
</tr>
<tr>
<td>IL-11</td>
<td>27.5</td>
<td>1.72</td>
<td>2.33</td>
</tr>
<tr>
<td>IL-12P35</td>
<td>27.8</td>
<td>2.04c</td>
<td>1.93</td>
</tr>
<tr>
<td>IL-12P40.1</td>
<td>29.2</td>
<td>1.27</td>
<td>0.75</td>
</tr>
<tr>
<td>IL-12P40.2</td>
<td>27.3</td>
<td>1.27</td>
<td>1.19</td>
</tr>
<tr>
<td>IL-12P40.3b</td>
<td>22.7</td>
<td>1.53</td>
<td>3.04c</td>
</tr>
<tr>
<td>iNOS b</td>
<td>25.5</td>
<td>8.08c</td>
<td>0.74</td>
</tr>
<tr>
<td>Arginase 1</td>
<td>30.2</td>
<td>1.31</td>
<td>0.79</td>
</tr>
<tr>
<td>Arginase 2b</td>
<td>21.4</td>
<td>1.33</td>
<td>3.08b</td>
</tr>
<tr>
<td>CXCa</td>
<td>24.0</td>
<td>1.56c</td>
<td>0.91</td>
</tr>
<tr>
<td>CXCb</td>
<td>29.5</td>
<td>1.17</td>
<td>1.00</td>
</tr>
<tr>
<td>CXCR1b</td>
<td>27.3</td>
<td>0.73</td>
<td>7.18c</td>
</tr>
<tr>
<td>CXCR2b</td>
<td>25.8</td>
<td>1.26</td>
<td>3.46c</td>
</tr>
<tr>
<td>MHC-II DAB1-2</td>
<td>18.1</td>
<td>0.94</td>
<td>0.91c</td>
</tr>
<tr>
<td>MHC-II-DAB3-4</td>
<td>28.2</td>
<td>1.50</td>
<td>1.32</td>
</tr>
<tr>
<td>NILT1</td>
<td>31.8</td>
<td>1.07</td>
<td>2.05c</td>
</tr>
<tr>
<td>NILT2b</td>
<td>22.3</td>
<td>1.22c</td>
<td>0.81c</td>
</tr>
</tbody>
</table>

a Take-off value is the point 80% before the peak of the second derivative of the raw data (Rotor-Gene Analysis Software, version 5.0).

b Significant difference compared with the LPS- and cAMP-stimulated cells.

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NO₃⁻, H₂O₂, and NO₂⁻) can oxidize the nonfluorescent DHR into the highly green fluorescent rhodamine 123, which localizes in the mitochondria (34). DHR is not oxidized by O₂⁻ (35, 36). The radical O₂⁻ can, however, react with other molecules to form radicals that can be measured by DHR (35). The low radical production by freshly isolated HKL could be ascribed, based on FSC-H/SSC-H, primarily to neutrophilic granulocytes and, to a lesser extent, to monocytes. In contrast, we observed a very high spontaneous radical production in carp head kidney-derived macrophages. Also, when radical production was measured using reduction of NBT, similar results were observed. In fact, for freshly isolated HKL, 90-min incubation was needed to obtain a substantial amount of NBT reduction, while head kidney-derived macrophages accounted for two times higher NBT reduction within 30 min only (unpublished data). Most likely, this high basal activity is caused by dead cells and/or cytokines also present in the cell cultures. In conclusion, cultured carp head kidney-derived macrophages provide us with an excellent model to investigate the polarization and functional heterogeneity of fish macrophages.

There is a growing interest in the role and functioning of alternatively activated macrophages. Recently, we described the possible conservation of alternative macrophage activation down to teleost fish (16). In the present study, we used cAMP to stimulate arginase activity, as a measure of alternative activation. Both NO production and arginase activity were higher in head kidney-derived macrophages than in freshly isolated HKL. Furthermore, we observed a clear increase of NO production or arginase activity after stimulation with LPS or cAMP, respectively. This increase in activity suggested that our culture system leads to enrichment for macrophages that are still able to polarize into either innate activated macrophages or alternatively activated macrophages.

For fish macrophages, it may be difficult to distinguish between innate and classically activated macrophages because LPS sensitivity and subsequent intracellular signaling are recognized to be different from the mammalian situation (37). Fish cells not only require high amounts of LPS for stimulation but also seem to lack several proteins involved in the LPS signaling pathway (37). In fish, LPS is believed to signal via β₂ integrins and not through TLR4 (37). True classical activation of macrophages would also require stimulation with IFN-γ. Previous experiments in our laboratory, using macrophage-activating factor (supernatants from mitogen-stimulated cells, believed to contain IFN-γ or IFN-γ-like proteins) in addition to LPS, did not result in an additional stimulation of nitrite production, neither in cultured nor in freshly isolated macrophages. Therefore, we believe the present NO response is due to innate rather than classical activation. Recently, the first fish IFN-γ sequences were identified and characterized (38, 39) and, in time, production of recombinant fish IFN-γ might allow for a further distinction between innate and classical activation of fish macrophages.

To further assess the polarization of head kidney-derived macrophages, we measured the gene expression of an array of immune-relevant genes after differential stimulation. Gene expression profiles of caMF and aaMF in both human and mouse are relatively well studied and extensively reviewed by Mantovani et al. (3, 40). From the differentially expressed genes in carp, TNF-α, IL-10, iNOS, CXCα, and NILT2 were significantly higher expressed in LPS-stimulated head kidney-derived macrophages. IL12P40.3, arginase 2, CXCR1, and CXCR2 were significantly higher expressed in cAMP-stimulated head kidney-derived macrophages. The up-regulation of TNF-α and iNOS after LPS stimulation (innate activation) is similar to the mammalian situation. It is more difficult to compare the CXC gene expression with the mammalian situation since phylogenetic analyses could not assign carp CXC chemokines according to the human CXC nomenclature (41). CXCL8–11 and CXCL-16 are typically up-regulated in mammalian caMF; similarly, CXCα was up-regulated in the LPS-stimulated head kidney-derived macrophages. The, in comparison, higher gene expression of IL-10 in LPS-stimulated macrophages could be primarily ascribed to a down-regulation of IL-10 in cAMP-stimulated macrophages. This is different from the mammalian situation. The expression of IL-10 in fish certainly requires further study (42). The expression of IL-12 is difficult to compare

**FIGURE 5.** Gene expression of NILT1 (A and B) and NILT2 (C and D) in head kidney (organ) of T. carassii-infected carp (A and C), or T. borreli-infected carp (B and D). A number of n = 32 carp were i.p. infected with 10,000 T. carassii per fish, and a number of n = 20 carp were i.p. infected with 10,000 T. borreli per fish. At wk 0, the week of infection, n = 4–5 carp were sacrificed as uninfected control fish. At every sample point, n = 4 fish from the T. carassii and n = 5 fish from the T. borreli infection were sacrificed, and head kidneys were removed, snap frozen in liquid nitrogen, and stored at −80°C. Gene expression was measured by means of real-time quantitative PCR and is shown relative to the gene expression of 40S. Averages and SD of n = 4–5 fish are given. *, p < 0.05, by Student’s t test, compared with uninfected control.
between different species since expression and translation of IL-12p35 and IL-12p40 are tightly, but differently, regulated even between human and mouse, as reviewed by Trinchieri (43). Expression can be regulated by both classical activators such as IFN-γ but also by alternative activators such as IL-4 and IL-13. In the present study, we describe an up-regulation of IL-12p40.3 gene expression after stimulation with cAMP, an alternative activator of fish macrophages. The up-regulation of arginase, CXCR1, and CXCR2 after cAMP stimulation (alternative activation) is comparable to the mammalian situation where CXCR1 and CXCR2 gene expression are up-regulated in alternatively activated macrophages (3). Increased arginase expression in alternatively activated macrophages confirms our previous findings (16); the increased CXCR1 and CXCR2 gene expression is, however, a new finding. We suggest that CXCR1 and CXCR2 might be useful new surface markers for alternative macrophage activation in fish.

This is the first report on a differential expression of NILT genes. The recently described NILT genes show significant similarity to the human triggering receptors expressed on myeloid cells (TREM) to CMRF35 and to the natural cytotoxicity receptor Nkp44. The location of zebrafish NILT homologs on chromosome 1 at 7 Mb downstream of the MHC class I cluster suggests that the carp NILT genes are related to either the Nkp44 or the TREM genes (20). Expression of Nkp44 is restricted to NK cells (44), whereas TREM are expressed on different cells of the myeloid lineage (45–48). Preliminary studies of NILT expression on carp peripheral blood lymphocytes were inconclusive as to ascribe a function to these genes (20). In the present study, we show both NILT1 and NILT2 expression in head kidney–derived macrophages and cells of the myeloid lineage, suggesting a close relationship to TREM.

The TREM gene family comprises at least six members of which TREM-1 and -2 are best studied. TREM-1 expression is up-regulated in myeloid cells after LPS stimulation (45) or during sepsis (49). TREM-2 expression is associated with regulating myeloid lineage development (50). TREM-2 is also believed to play a role in chronic inflammation and may stimulate production of constitutive rather than inflammatory chemokines and cytokines (45, 46). Typically, aMφ are associated with chronic inflammation (51). In parallel, TREM-2 was shown to be up-regulated during chronic infections of mice with African trypanosomes (52), together with other markers, for alternative activation (53, 54).

We have shown an up-regulation of NILT1 in head kidney–derived macrophages stimulated with cAMP, an inducer of alternative activation in carp macrophages (16). Furthermore, NILT1 gene expression was up-regulated during the later time points of infection with T. carassius. Recent in vivo experiments suggest that, during infection with T. carassius, macrophages are predominantly alternatively activated during the late phase of infection (J. M. Forlenza, C. M. S. Ribeiro, B. de Vries, H. F. J. Savelkoul, and G. F. Wiegerijtes, submitted for publication). In addition, NILT1 gene expression was hardly up-regulated during an infection with T. borrelli, where macrophages are primarily innate activated (10, 11). Currently we are developing specific Abs against the NILT and CXCR proteins to unequivocally assign these proteins as surface markers of fish macrophages. We suggest that the NILTs might be TREM homologs and that NILT1, together with CXCR1 and CXCR2, could likely be used as novel surface markers for alternatively activated macrophages in fish.

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Disclosures
The authors have no financial conflict of interest.

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