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The Thyroid Hormone Triiodothyronine Controls Macrophage Maturation and Functions

Protective Role during Inflammation

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The endocrine system participates in regulating macrophage maturation, although little is known about the modulating role of the thyroid hormones. *In vitro* results demonstrate a negative role of one such hormone, triiodothyronine (T_3), in triggering the differentiation of bone marrow–derived monocytes into unpolarized macrophages. T_3-induced macrophages displayed a classically activated (M1) signature. A T_3-induced M1-priming effect was also observed on polarized macrophages because T_3 reverses alternatively activated (M2) activation, whereas it enhances that of M1 cells. *In vivo*, circulating T_3 increased the content of the resident macrophages in the peritoneal cavity, whereas it reduced the content of the recruited monocyte-derived cells. Of interest, T_3 significantly protected mice against endotoxemia induced by lipopolysaccharide i.p. injection; in these damaged animals, decreased T_3 levels increased the recruited (potentially damaging) cells, whereas restoring T_3 levels decreased recruited and increased resident (potentially beneficial) cells. These data suggest that the anti-inflammatory effect of T_3 is coupled to the modulation of peritoneal macrophage content, in a context not fully explained by the M1/M2 framework. Thyroid hormone receptor expression analysis and the use of different thyroid hormone receptor antagonists suggest thyroid hormone receptor β1 as the major player mediating T_3 effects on macrophages. The novel homeostatic link between thyroid hormones and the pathophysiological role of macrophages opens new perspectives on the interactions between the endocrine and immune systems. *(Am J Pathol 2014, 184: 230–247; http://dx.doi.org/10.1016/j.ajpath.2013.10.006)*

Accumulating evidence indicates that the endocrine and immune systems engage in a complex cross talk. Hormones and endocrine transmitters bind to immune system cells, leading to production of factors that modify immune cell functions and tune immune responses.[1] Several studies have addressed the role of different hormones, including growth hormone, leptin, insulin-like growth factor-1, steroid hormones, thyroid-stimulating hormone, prolactin, and neurohypophyseal hormones, in the immune system.[1–5] Comparatively, our understanding of the role, as immune-modulating factors, of the thyroid hormones (THs) 3,3′,5′-triiodo-L-thyronine (T_3) and L-thyroxine (T_4), is still rather incomplete,[6] despite the significant role played by these molecules in complex function in which the immune system participates, including differentiation, growth, and metabolism.[7,8] Reports focusing mainly on natural killer cells, effector B and T lymphocytes, and dendritic cells revealed that THs may support the basal functions of immune cells, although contradictory results are reported regarding the effect of hypohyperthyroidism on immunity.[6,9–12] In particular, scant information exists about the role of THs in modulating the functions of macrophages, despite the fact

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that these monocyte-derived immune-competent cells play key roles in innate and adaptive immunity[13,14] and that THs may change the level of cytokines involved in their function.[6] The magnitude of macrophage inflammatory response is stimulated in rats during the hypothyroid condition and inhibited during the hyperthyroid condition; in addition, in hyperthyroid rats, phagocytic activity and hydrogen peroxide release by macrophages are suppressed.[15] In hypothyroid rats, conversely, macrophage phagocytosis and reactive oxygen species release are both enhanced.[16] T_4 and, in some cases, T_3 were shown to stimulate mouse macrophage phagocytosis.[17–20] THs were also suggested to increase mouse peritoneal macrophage chemotaxis *in vitro*.[21] Recently, a beneficial role of THs in macrophage-mediated meningococcal infection has also been demonstrated.[20]

Macrophages perform an important immune surveillance role through their ability to sense and adapt to local microenvironmental signals.[13] Two distinct states of polarized activation for macrophages have been recognized: the classically activated (M1) macrophage phenotype and the alternatively activated (M2) macrophage phenotype.[13,22,23] The M1 macrophages are activated by Toll-like receptor ligands, such as lipopolysaccharide (LPS) and interferon-γ (IFN-γ), express proinflammatory cytokines, mediate defense of the host from a variety of bacteria, protozoa, and viruses, and have roles in antitumor immunity. The M2 macrophages are stimulated by IL-4 or IL-13 and have anti-inflammatory, protumoral function and regulate wound healing.[13,23,24]

The classic genomic actions of THs are mediated by ligand-inducible transcription factors that are members of the steroid/thyroid hormone receptor superfamily. There are two types of TH nuclear receptors (TRs) encoded by TRα and TRβ genes.[7,25] TRα has one T_3-binding splice product, *TRα1*, and two non–T_3-binding splice products, *TRα2* and *TRα3*, with several additional truncated forms. *TR*β has three major T_3-binding splice products: *TR*β*1, TR*β*2*, and *TR*β*3*. The expression of the mRNAs coding for TRα and TRβ has been reported in bone marrow–derived macrophages[26]; however, the presence of TR proteins and the effects of TH on macrophage differentiation and M1/M2 macrophage activation are unknown.

In the present study, we have investigated the role of the T_3 system in the regulation of growth/development and functional phenotype of unpolarized macrophages, as well as its influence on M1 or M2 activation. We demonstrate that macrophages, at different stages of growth, express the mRNAs of the two major TR isoforms, TRα1 and TRβ1, but only TRβ1 was detected at the protein level. The action of T_3 at intracellular TRs (likely at TRβ1) consists of the regulation of unpolarized macrophage, M1, or M2 development. By comparative analysis of euthyroid and hypothyroid animals, we also found that this function by T_3 is reflected in the profile of peritoneal macrophages, indicating the functional relevance of the interactions of TH/macrophages in basal conditions and during systemic inflammation.

Materials and Methods

Animals

Experiments were performed on C57BL/6J female mice at 12 to 18 weeks after birth (20 to 30 g body weight). Animals were kept in a regulated environment (23°C ± 1°C, 50% ± 5% humidity) with a 12-hour light/dark cycle (lights on at 8 AM). All studies were conducted in accordance with the Italian law on animal care number 116/1992 and the European Communities Council Directive EEC/609/86. The experimental protocols were also approved by the Ethics Committee of the University of Milan (Milan, Italy). All efforts were made to reduce both animal suffering and the number of animals used.

Isolation of Bone Marrow–Derived Cells, Differentiation, and Activation of Macrophage Primary Cultures

By using published protocols,[27,28] bone marrow precursors from the femur and tibia of mice were isolated and propagated for 8 days in α-minimum essential medium (37°C, 5% CO_2 in a humidified atmosphere) containing 10% fetal bovine serum (FBS) in the presence of 100 ng/mL macrophage-specific colony-stimulating factor (M-CSF) to generate macrophages (Supplemental Figure S1). Adherent cells were then collected and cultured in the presence of 10 ng/mL M-CSF to form monolayers of differentiated, unpolarized macrophages. Cells were cultured for 2 additional days in the presence of 50 ng/mL IFN-γ to generate activated, polarized M1 cells[24] and for 4 additional days with 10 ng/mL IL-4 and 10 ng/mL M-CSF to generate activated, polarized M2a cells.[24] In agreement with previous reports,[28,29] the morphological characteristics of M1 cells appeared flattened and rounded, with different cells displaying a fried egg–like shape, whereas M2 cells were more stretched and elongated (spindle-like morphological features) (Supplemental Figure S2A). Unpolarized macrophage cells appeared adherent, with the characteristic intermediate morphological characteristics of polarized macrophages, with some cells being more elongated, whereas others were more rounded.

In Vitro T_3 Treatments

The thyroid gland produces mainly T_4, but other tissues deiodinate it to the more potent hormone, T_3. In our experiments, T_3 or, when indicated, the integrin αVβ3 antagonist, tetraiodothyroacetic acid (tetrac; Sigma-Aldrich, St. Louis, MO), and the TR antagonist, 1-850, were added daily to the medium. Parallel cultures were maintained with T_3 vehicle and used as a control. During treatments, all cells were exposed to 10% TH-depleted FBS-containing medium.[7] Routinely, TH-depleted FBS-containing medium was added 1 to 2 days before T_3 treatment.[30] TH-depleted FBS was obtained by adsorption of FBS onto analytical-grade anion exchange resin (AG 1-X8 Resin, 200 to 400 mesh,

Table 1 Antibodies and Dilutions Used in Flow Cytometry Analysis, Western Blot Analysis, and Immunofluorescence Microscopy of Macrophages

Primary antibody	Isotype	Dilution	Secondary antibody	Dilution
PE hamster polyclonal anti-CD11c: *117308*	IgG	1:100 (FC)	—	—
APC rat monoclonal anti-CD14: *560634*	IgG1	1:100 (FC)	—	—
APC hamster polyclonal anti-CD34: *128612*	IgG	1:100 (FC)	—	—
FITC rat monoclonal anti-CD68: *SM1550FS*	IgG2a	1:250 (FC)	—	—
PE rat polyclonal anti-CD206: *141706*	IgG2a	1:100 (FC)	—	—
FITC rat monoclonal anti-F4/80: *BM4008FS*	IgG2b	1:500 (FC), 1:50 (IF)	—	—
Rabbit polyclonal anti-TRα/β: *sc-772*	IgG	1:500 (WB)	HRP—anti-rabbit	1:2000
Goat polyclonal anti-TRα1: *sc-10819*	IgG	1:50 (IF)	Alexa Fluor 546 anti-goat	1:500
Rabbit polyclonal anti-TRβ: *ab53170*	IgG	1:500 (WB)	HRP—anti-rabbit	1:2000
Mouse monoclonal anti-TRβ1: *sc-738*	IgG	1:50 (IF, FC)	Alexa Fluor 546 anti-mouse	1:500
Rabbit polyclonal anti-arginase 1: *sc-20150*	IgG	1:5000 (WB)	AP—anti-rabbit	1:10,000
Mouse monoclonal anti—β-actin: *A5441*	IgG1	1:10,000 (WB)	HRP—anti-mouse	1:5000

AP, alkaline phosphatase; APC, allophycocyanin; FC, flow cytometry; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; IF, immunofluorescence; PE, phycoerythrin; WB, Western blot analysis.

chloride form), according to the manufacturer-recommended procedure (Bio-Rad, Hercules, CA).

Immunophenotyping by Flow Cytometry

To determine the expression of several cell surface markers on macrophages, a fluorescence-activated cell sorter analysis was performed. In brief, collected cells were washed with PBS supplemented with 2% FBS and incubated with different antibodies for 1 hour at 4°C. After washing, the extent of marker expression in duplicate samples was analyzed using an FC500 Dual Laser system (Beckman Coulter, Brea, CA). Unstained, single stains, and fluorescence minus one controls were used for setting compensation and gates. Irrelevant isotypic monoclonal antibodies were routinely used as negative controls. FCS Express, version 3 (De Novo System, Portland, OR), was then used for data analysis. When indicated, mean fluorescence intensity (MFI; arbitrary units) was determined as a measure of the extent of marker cell surface expression, excluding cells negative to the staining.

As listed in Table 1, the cell markers used were F4/80, a member of the epidermal growth factor—transmembrane 7 family, CD68 (macrosialin), a member of the lysosomal-associated membrane protein family with a macrophage-specific mucin-like extracellular domain, the integrin α X chain protein CD11c (CR4), CD14, a glycosylphosphatidyl inositol—linked membrane glycoprotein, the mannose receptor 1 CD206, and CD34, a member of a family of single-pass transmembrane proteins. F4/80, CD68 (conventional pan-macrophage markers),[13,31,32] and CD11c (conventional dendritic cell marker) are commonly used to distinguish macrophages from dendritic cells.[13,33,34] CD14 and CD206 are highly expressed in M1 or M2a cells, respectively,[24] whereas CD34 shows distinct expression on early hematopoietic precursors and vascular-associated tissue.[35] Quantitative image analysis revealed that the percentage of cells that positively immunostained for F4/80 ($F4/80^+$) was approximately 97% to 98% of total adherent cells (Supplemental Figure S2B). Similar results were achieved with CD68, whereas the levels of $CD14^+$ and $CD206^+$ cells were low. In contrast, $CD11c^+$ and $CD34^+$ cells were almost undetectable. These results confirm the macrophage phenotype (unpolarized) and the high purity of our unpolarized macrophage cell preparation obtained from bone marrow—derived cells. Quantitative image analysis also revealed that, similar to unpolarized macrophages, the percentage of cells that positively immunostained for F4/80 was high in both M1 and M2 cells. However, the levels of $CD14^+$ cells were high in the presence of IFN-γ and almost undetectable in the presence of IL-4. Conversely, the levels of $CD206^+$ cells were high in the presence of IL-4 and almost undetectable in the presence of IFN-γ. Thus, the activated, polarized phenotype of M1 cells was $F4/80^+$/$CD14^+$/$CD206^-$ and that of M2 cells was $F4/80^+$/$CD14^-$/$CD206^+$, in agreement with previous reports.[24]

PCR Experiments

Total RNA from unpolarized macrophage, M1, and M2 cells was extracted with the High Pure RNA Isolation Kit (Roche Applied Science, Mannheim, Germany), according to the manufacturer-recommended procedure. After solubilization in RNase-free water, total RNA was quantified by the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). First-strand cDNA was generated from 1 μg of total RNA using the ImProm-II Reverse Transcription System (Promega, Madison, WI). As show in Table 2, a set of primer pairs, amplifying fragments ranging from 85 to 354 bp, was designed to hybridize to unique regions of the appropriate gene sequence. The PCRs were performed using 1 μL of cDNA and the GoTaq Green Master Mix (Promega), containing 500 nmol/L of appropriate primers. The amplification reactions were performed in the MJ Mini personal thermal cycler (Bio-Rad). A sample (5 μL) of the PCR was electrophoresed on ethidium

Table 2 Primer Pairs Designed for PCR Analysis

Name	Symbol	Gene accession No.	Primer sequence	Amplicon (bp)	Source
TRα	*Thra*	NM_178060	F: 5′-GGACAAAGACGAGCAGTGTGTCG-3′ R: 5′-TGTGCGGCGAAAGAAGCCCT-3′	100	Custom designed
TRβ1	*Thrb*	NM_001113417	F: 5′-CAGAAGCCCCGTCCAGACCGA-3′ R: 5′-TTCAGTGCACCACGCCTCTCC-3′	101	Custom designed
CCL2	*Ccl2*	NM_011333	F: 5′-AGGTGTCCCAAAGAAGCTGTA-3′ R: 5′-ATGTCTGGACCCATTCCTTCT-3′	85	RTPrimerDB*
CCL5	*Ccl5*	NM_013653	F: 5′-ATATGGCTCGGACACCACTC-3′ R: 5′-GTGACAAACACGACTGCAAGA-3′	123	RTPrimerDB*
CCL9	*Ccl9*	NM_011338	F: 5′-CCCTCTCCTTCCTCATTCTTACA-3′ R: 5′-AGTCTTGAAAGCCCATGTGAAA-3′	141	PrimerBank†
CD36	*Cd36*	NM_001159558 NM_007643 NM_001159555 NM_001159557 NM_001159556	F: 5′-ATGGGCTGTGATCGGAACTG-3′ R: 5′-GTCTTCCCAATAAGCATGTCTCC-3′	110	PrimerBank†
CXCL9	*Cxcl9*	NM_008599	F: 5′-TCCTTTTGGGCATCATCTTCC-3′ R: 5′-TTTGTAGTGGATCGTGCCTCG-3′	110	PrimerBank†
CXCL10	*Cxcl10*	NM_021274	F: 5′-TCCTTGTCCTCCCTAGCTCA-3′ R: 5′-ATAACCCCTTGGGAAGATGG-3′	124	RTPrimerDB*
CXCL12	*Cxcl12*	NM_001012477 NM_013655 NM_021704	F: 5′-TGCATCAGTGACGGTAAACCA-3′ R: 5′-TTCTTCAGCCGTGCAACAATC-3′	146	PrimerBank†
CXCL16	*Cxcl16*	NM_023158	F: 5′-CCTTGTCTCTTGCGTTCTTCC-3′ R: 5′-TCCAAAGTACCCTGCGGTATC-3′	139	PrimerBank†
CXCR4	*Cxcr4*	NM_009911	F: 5′-TCCAACAAGGAACCCTGCTTC-3′ R: 5′-TTGCCGACTATGCCAGTCAAG-3′	101	RTPrimerDB*
IL-1β	*Il1b*	NM_008361	F: 5′-GCAACTGTTCCTGAACTCAACT-3′ R: 5′-ATCTTTTGGGGTCCGTCAACT-3′	89	PrimerBank†
IL-10	*Il10*	NM_010548	F: 5′-GCTCTTACTGACTGGCATGAG-3′ R: 5′-CGCAGCTCTAGGAGCATGTG-3′	105	PrimerBank†
IL-13	*Il13*	NM_008355	F: 5′-AGACCAGACTCCCCTGTGCA-3′ R: 5′-TGGGTCCTGTAGATGGCATTG-3′	123	RTPrimerDB*
TNF-α	*Tnf*	NM_013693	F: 5′-TTCTGTCTACTGAACTTCGGGGTGATCGGTCC-3′ R: 5′-GTATGAGATAGCAAATCGGCTGACGGTGTGGG-3′	354	RTPrimerDB*
GAPDH	*Gapdh*	NM_008084	F: 5′-ACCCAGAAGACTGTGGATGG-3′ R: 5′-ACACATTGGGGGTAGGAACA-3′	172	RTPrimerDB*

*Available at *http://medgen.ugent.be/rtprimerdb/index.php*, last accessed February 26, 2013.
†Available at *http://pga.mgh.harvard.edu/primerbank*, last accessed February 26, 2013.
F, forward; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; R, reverse.

bromide—containing 2.5% agarose gel by the use of the Bio-Rad Subcell GT System. After migration, bands corresponding to the amplified products were visualized with the Bio-Rad Gel Doc XR System. Real-time quantitative PCR (qPCR) was performed using a LightCycler 480 SYBR Green I Master (Roche Applied Science) on Roche LightCycler 480 Instrument, according to the manufacturer-recommended procedure. All reactions were run as triplicates. The melt-curve analysis was performed at the end of each experiment to verify that a single product per primer pair was amplified. Regarding control experiments, gel electrophoresis was also performed to verify the specificity and size of the amplified qPCR products. Samples were analyzed using the Roche LightCycler 480 Software release 1.5.0 and the second derivative maximum method. The fold increase or decrease was determined relative to a calibrator after normalizing to glyceraldehyde-3-phosphate dehydrogenase (internal standard) through the use of the following formula:

$2^{-\Delta\Delta C_T}$.[36,37]

Because there is a great overlap in surface protein expression between the different macrophage subsets, we decided to quantify specific gene expression profiles.[13] Thus, macrophage activation obtained under our experimental protocol was confirmed by qPCR of specific patterns of cytokines and receptors. In agreement with previous reports,[27] M1 cells, when compared with M2 cells, expressed higher levels of transcripts encoding chemokine (C-C motif) ligand (CCL) 5 (alias RANTES, regulated upon activation, normal T cell expressed and secreted), *CXCL9* (alias monokine induced by IFN-γ), *CXCL10* (alias IFN-γ—induced protein-10), *CXCL16*, *IL-1*β, *IL-10*, and *TNF-*α (Supplemental Figure S3A). Conversely, M2 cells, when compared with M1, expressed higher levels of the transcripts encoding

CCL2, CCL9, the integral membrane protein CD36, the chemokine receptor CXCR4 (alias fusin or CD184), and IL-13. The melt-curve analysis and the conventional gel electrophoresis confirmed the qPCR readings (Supplemental Figure S3, B and C). These results indicate that IFN-γ–treated cells displayed a typical M1 gene signature and IL-4–treated cells had a typical M2 gene signature.[13,22–24]

Western Blot Analysis

Unpolarized macrophage, M1, and M2 cells were harvested and homogenized for 10 minutes at 4°C in radioimmunoprecipitation assay lysis buffer, containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 1 mmol/L EDTA, 0.1% SDS, and supplemented with protease inhibitors. The supernatant was obtained by centrifugation at 2000 × *g* (5 minutes). When indicated, human melanoma C32 whole cell lysate (*sc-2205*) (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a positive control, according to the manufacturer-recommended procedure. Protein concentration was determined using the bicinchoninic acid assay (Thermo Fisher Scientific), according to the manufacturer-recommended procedure. SDS and β-mercaptoethanol were added to samples before boiling, and an equal amount of proteins (40 μg per lane) was separated by 10% SDS-PAGE with the Bio-Rad Mini-PROTEAN 3 system.[38] After transfer of proteins onto nitrocellulose membrane (GE Healthcare, Milan, Italy), blots were blocked for 1 hour with Tris-buffered saline–0.1% (v/v) Tween containing 5% skimmed-milk powder. The incubation with primary antibody (Table 1) was performed overnight at 4°C. After 1 to 3 hours of incubation with the appropriate horseradish peroxidase (HRP)–conjugated secondary antibody, bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific), according to the manufacturer's instructions, and exposure to autoradiography Cl-Xposure films (Thermo Fisher Scientific) or with a Bio-Rad ChemiDoc MP imaging system. When indicated, alkaline phosphatase (AP)–conjugated secondary antibody was used in combination with the colorimetric AP and peroxidase substrate detection system (Sigma-Aldrich, St. Louis, MO), according to the manufacturer's instructions. To monitor potential artifacts in loading and transfer among samples in different lanes, the blots were routinely treated with the Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) and reprobed with anti–β-actin primary antibody and the appropriate HRP-conjugated secondary antibody (Table 1).

Immunofluorescence Microscopy and Flow Cytometry of Thyroid Hormone Receptors

By using published protocols,[39,40] unpolarized, M1, M2, and peritoneal macrophages (see later) were cultured on glass coverslips coated with poly-L-lysine. Cells were fixed in ice-cold 4% paraformaldehyde in PBS for 5 minutes and quenched with 1% glycine-PBS. Incubation with anti-F4/80 antibody (Table 1) was then performed in a humid chamber overnight at 4°C. Cells were then permeabilized in 0.25% Triton X-100 in PBS and incubated with anti-TRα1 and anti-TRβ1 antibodies for 2 hours before staining (1 hour) with appropriate secondary antibodies for fluorescence detection (Table 1). Nuclei were also stained with Hoechst 33258 for 5 minutes. Samples were washed in PBS and mounted on glass slides for fluorescence examination using a Leica DMI4000 B automated inverted microscope equipped with a DCF310 digital camera (Leica Microscopy Systems, Heerbrugg, Switzerland). Images were collected under a 100× oil immersion objective. Image acquisition was controlled by Leica LAS AF software version 2.5.0.6735.

The expression of TRβ1 on unpolarized macrophage cells was performed by flow cytometry, as previously described. Cells were incubated with anti-TRβ1 antibody (Table 1) for 2 hours before staining (1 hour) with appropriate secondary antibodies. For intracellular staining, cells were fixed in 4% paraformaldehyde and 0.1% saponin, which were added to the antibody and wash solutions because permeabilization with saponin is reversible.

Cell Counts

The number of viable adherent unpolarized macrophage cells was evaluated by dye exclusion (indicative of an intact membrane) using the DNA-binding probe, propidium iodide (PI) or trypan blue. Briefly, pelleted cells (unlysed) were added to PBS containing 2 μg/mL PI and cells were counted by flow cytometry within 30 minutes. In particular, cells were acquired for a constant amount of time, using enough time to acquire at least 10,000 events for samples. Alternatively, the cell concentration was measured after 0.2% trypan blue staining by counting trypan blue–excluding cells (aliquots of 50 μL) in a standard hemocytometer chamber under low-power microscopy.

Apoptosis

Apoptosis was analyzed as described previously.[41,42] Briefly, phosphatidylserine exposure on the outer leaflet of the plasma membrane in PI-excluding cells was detected by flow cytometry of unpolarized macrophage cells (duplicate samples) stained for 15 minutes with 1 g/mL fluorescein isothiocyanate–labeled annexin V.

Phagocytosis

The Phagocytosis Assay Kit (IgG fluorescein isothiocyanate) (Cayman Chemical Company, Ann Arbor, MI) was used following the manufacturer's instruction protocol. The kit uses latex beads coated with fluorescently labeled rabbit-IgG. The engulfed fluorescent beads in unpolarized macrophage, M1, and M2 cells were determined by flow cytometry at 3-hour bead exposure, when the number of M1

phagocytic cells reached the maximal values. M1 cells were also incubated with the latex beads for 1 hour (intermediate values of fluorescent-positive cells). Both the percentage of macrophages engaging in phagocytosis (percentage positive) and MFI (as a measure of the extent of fluorescence/phagocytosed latex beads) were determined. Because flow cytometry measures both beads bound to the surface and beads internalized, microscopy images on a Leica DMI4000 B automated inverted microscope equipped with a DCF310 digital camera were taken of cells after phagocytosis. After 3 hours of bead exposure, fluorescence appeared inside the M1 cells (data not shown), thus suggesting that phagocytosis had taken place. These results support that the uptake measured by flow cytometry is phagocytosis, although we cannot exclude the surface binding of beads.

Cytokine Release

By using published protocols,[27,39,43] IFN-γ, IL-13, and tumor necrosis factor (TNF)-α levels were measured and quantified in the culture cell supernatants by means of commercially available enzyme-linked immunosorbent assay (ELISA) kits (RayBiotech, Norcross, GA), according to the manufacturer-recommended procedure. Briefly, the supernatants of unpolarized macrophage cells were collected after 24 or 48 hours of culture, and the color intensity of the reaction product (proportional to the cytokine concentration) was quantified spectrophotometrically by the GloMax Multi detection system (Promega). Each experiment was performed in quadruplicate. At the end of each experiment, viable cells were counted with a standard hemocytometer chamber and trypan blue exclusion. Release data were expressed as pg/mL of medium/10^6 cells. Basal values were consistent with previous reports.[27,43]

Migration Assay

The migration of unpolarized macrophage cells was assessed using Costar Transwell permeable supports (Corning Incorporated Life Sciences, Tewksbury, MA), which are chambers with inserts (8-μm pore polycarbonate membranes). Following published protocols,[44,45] unpolarized macrophages were plated in the appropriate medium in the upper chamber, whereas medium with 100 ng/mL M-CSF (used as chemoattractant) was placed in the lower chamber. After 24-hour migration, nonmigratory cells were removed from the top of the insert membrane by gently wiping (cotton swabs). The underside of each membrane was fixed and stained with crystal violet, and the number of cells that migrated completely through the pores was counted per 0.1 mm^2 using a scored eyepiece.[28] From this, the total number of migrated cells was calculated.

Hypothyroid Animals

Mice were made hypothyroid by treatment (at least 4 weeks) with the 0.02% anti-thyroidal drug, methimazole, and 0.1% sodium perchlorate in the drinking water.[46] The treatment was continued until the end of experiments. In agreement with previous reports,[46–48] classic signs of hypothyroidism, such as reduced weight compared with the untreated controls (euthyroid mice), were observed. At the end of treatment, the blood of five mice per group (euthyroid and hypothyroid) was withdrawn from eyes of anesthetized animals for the assessment of thyroid status. Briefly, total T_3, total T_4, and thyrotropin (TSH) in serum specimens were measured by means of triiodothyronine/thyroxine ELISA kits (Calbiotech, Spring Valley, CA) and TSH ELISA kits (Bio-Medical Assay, Beijing, China), according to the manufacturer-recommended procedure. As shown in Table 3, the hormone levels of the animals mirrored the expected values,[49] in agreement with other reports in mice.[46,50–53]

Table 3 Serum Levels of THs and TSH in Mice, as Assessed by ELISA

Name	Unit	Euthyroid	Hypothyroid
Total T_3	nmol/L	2.19 ± 0.23	0.22 ± 0.02
Total T_4	nmol/L	63.19 ± 14.52	7.14 ± 3.93
TSH	mIU/L	9.43 ± 1.15	23.02 ± 5.66

When indicated, hypothyroid mice were treated i.p. with 0.2 μg/g body weight per day T_3 for 5 days, an approach used to increase the levels of circulating hormones, as previously described.[47,54] Hypothyroid mice were also i.p. injected with the TR antagonist, amiodarone hydrochloride (AMIO).[55] Each mouse received injections of AMIO at a dose of 20 μg/g body weight per day for 2 weeks before treatment with T_3, as previously described. Mice used as a control received i.p. injections of vehicle at an equivalent volume as the experimental groups. For all of the experiments and analytic methods, we used three mice per group as a minimum. All of the experiments were repeated at least three times.

Isolation of Resident Peritoneal Cavity Cells

The peritoneal cavity is a unique compartment within which a variety of immune cells reside, and from which macrophages are commonly drawn without altering their physiological properties.[56] Peritoneal macrophages from euthyroid/hypothyroid mice were collected by peritoneal lavage with 5 mL ice-cold PBS supplemented with 3% FBS. The exudates were strained to remove peritoneal debris and centrifuged to isolate the cell pellet. The cell pellet was immediately resuspended in ice-cold PBS and kept on ice until immunophenotyping by flow cytometry.

In Vivo Inflammation Experiments

As previously reported,[45,57] acute illness was induced by a single i.p. injection of 10 to 15 μg/g body weight of LPS (endotoxin, *Escherichia coli* serotype O55:B5). Survival

was monitored for 96 hours. In another set of experiments, mice received a single injection i.p. of LPS for 16 hours before the isolation and flow cytometry analysis of resident peritoneal cavity cells.

Statistics

On verification of normal distribution, statistical significance of raw data between the groups in each experiment was evaluated using the Student's *t*-test or one-way ANOVA, followed by the Newman-Keuls multiple comparison post test. Kaplan-Meier data were analyzed with the multiple comparison survival curve method using the log-rank (Mantel-Cox) test. The GraphPad Prism software package version 5.01 (GraphPad Software, San Diego, CA) was used. After statistics, data belonging from different experiments were represented and averaged in the same graph. The results were expressed as means ± SEM of the indicated *n* values.

Chemicals

M-CSF, IFN-γ, and IL-4 were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). α-Minimum essential medium and annexin V were obtained from Life Technologies (Monza, Italy). FBS and PBS were purchased from Euroclone (Milano, Italy). Primer pairs were obtained from Primm (Milano, Italy). The 1-850 was obtained from Santa Cruz Biotechnology. Antibodies are as follows (Table 1): CD11c, CD34, and CD206 (BioLegend, San Diego, CA), CD14 (BD Biosciences, Franklin Lakes, NJ), CD68 and F4/80 (Acris Antibodies, Herford, Germany), TRβ (*ab53170*) (Abcam, Cambridge, UK), TRα/β (*sc-772*), TRα1 (*sc-10819*), TRβ1 (*sc-738*), and arginase 1 (*sc-20150*) (Santa Cruz Biotechnology), β-actin (*A5441*) (Sigma-Aldrich), HRP—anti-rabbit (Cell Signaling, Danvers, MA), AP—anti-rabbit (Promega), HRP—anti-mouse (Bio-Rad), and Alexa Fluor 456 anti-goat/mouse/rabbit (Life Technologies). When appropriate, all reagents and solutions were verified to be endotoxin free by the Sigma Limulus amebocyte lysate assay (sensitivity limit, 0.1 ng/mL). Where not specified, chemicals and reagents were purchased from Sigma-Aldrich.

Results

TR Expression in Unpolarized Macrophage Cells

The major isoforms, TRα1 and TRβ1, are bona fide receptors to which T_3 binds with high affinity.[7] TRs were found to be widely expressed throughout the body, including in immune and hematopoietic cells.[7,11,12,26,58–61] As shown in the PCR experiments of Figure 1A, primary cultures of unpolarized macrophage cells express detectable mRNA levels of TRα and TRβ1. In addition, as depicted in Western blot experiments with anti-TRα/β antibody (Figure 1B), using C32 cell proteins as a positive control, we detected two immunoreactive bands corresponding to the molecular weights of TRα1 (47 kDa) and TRβ1 (52 kDa). In contrast, we detected only the TRβ-specific band in unpolarized macrophage cells. In another set of experiments, immunofluorescence analysis of unpolarized macrophage cells with anti-TRα1 and anti-TRβ1 antibodies, in combination with an anti-F4/80 antibody (a macrophage-specific surface marker) and Hoechst staining, allowed us to study the expression of TRs at the cellular level. In broad

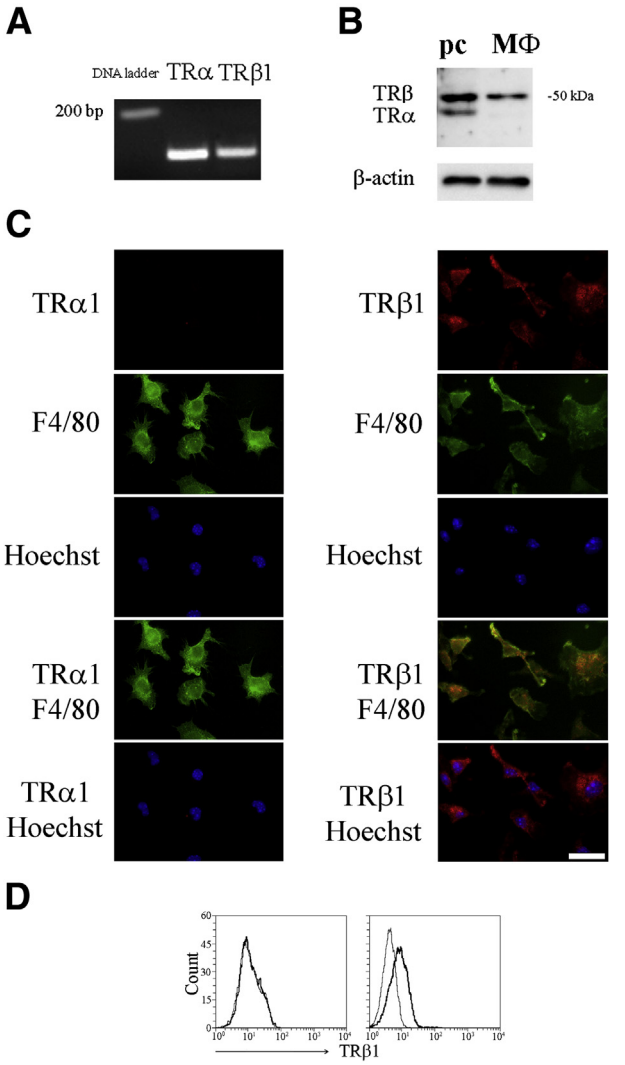

Figure 1 TR expression in unpolarized macrophage (MΦ) cells. **A**: PCR products of distinct TR mRNAs in unpolarized macrophage cells after 8 days of culture. The panel is representative of four independent experiments. **B**: Western blot analysis of TR expression performed with the anti-TRα/β (*sc-772*) antibody. β-Actin (anti—β-actin *A5441*) was used as an internal standard. The image is representative of four independent experiments. pc, positive control. **C**: Immunofluorescence analysis of unpolarized macrophage cells with anti-TRα1 (*sc-10819*) and anti-TRβ1 (*sc-738*) antibodies (red). Unpolarized macrophage cells were also stained with the anti-F4/80 antibody (green) and with Hoechst dye (blue). The images are representative of three independent experiments. Scale bar = 20 μm. **D**: Flow cytometry analysis of TRβ1 (*sc-738*) expression in unpolarized macrophage cells either left intact (**left panel**) or permeabilized (**right panel**). Results are representative of three independent experiments.

agreement with Western blot analysis, we did not find a TRα1-specific signal (Figure 1C). Conversely, the immunostaining for TRβ1 showed bright cytoplasmic/perinuclear labeling, whereas nuclear staining was scarce and diffuse. A similar expression pattern of TRβ1 staining has been already demonstrated in mouse dendritic cells.[11] As shown in the analysis of TRβ1 expression by flow cytometry (Figure 1D), no signal was detected in intact unpolarized macrophage cells, whereas permeabilized cells exhibited specific staining for TRβ1, further confirming the intracellular distribution of this molecule.

Inhibitory Effects of T_3 on Macrophage Differentiation

To investigate the role of THs in the physiological characteristics of macrophages, and their influence on macrophage development, we investigated whether T_3 affects the differentiation of monocytes into unpolarized macrophages. T_3 was applied daily beginning from the isolation of bone marrow–derived cells. In agreement with previous *in vitro* studies on brain macrophages,[47] T_3 was added at a final concentration of 500 nmol/L, which is a supraphysiological concentration in mouse,[50–52] (Table 3) giving maximal TRα/TRβ occupancy.[62] As shown in Figure 2, A and B, after 8 days of continuous T_3 application, the number of adherent viable cells decreased by almost 35% and 44% versus control cells, as assessed by the trypan blue and PI exclusion tests, respectively. Actions of thyroid hormone that are not initiated by binding of the hormone to intranuclear TRs are termed nongenomic.[63] Plasma membrane-initiated actions begin at a receptor on integrin αVβ3. Tetrac is a TH analogue that inhibits binding of iodothyronines to the integrin receptor and is a probe for the participation of this receptor in cellular actions of the hormone.[30,63,64] The simultaneous application of 500 nmol/L tetrac and T_3 did not modify T_3 effects on the number of adherent viable cells, whereas the addition of 1 μmol/L TR antagonist 1-850[65–67] inhibited T_3 actions (Figure 2A). In addition, T_3 effects were dependent on the time of exposure (Figure 2B). The absence of phosphatidylserine exposure on the outer leaflet of the plasma membrane, measured by annexin V staining, excluded the induction of apoptosis during T_3 exposure (Supplemental Figure S4). Similarly, flow cytometry analysis revealed that T_3 reduced the expression of the pan-macrophage markers F4/80 and CD68 and the number of F4/80$^+$ cells in the population of adherent cells (Figure 2, C and D), suggesting that not

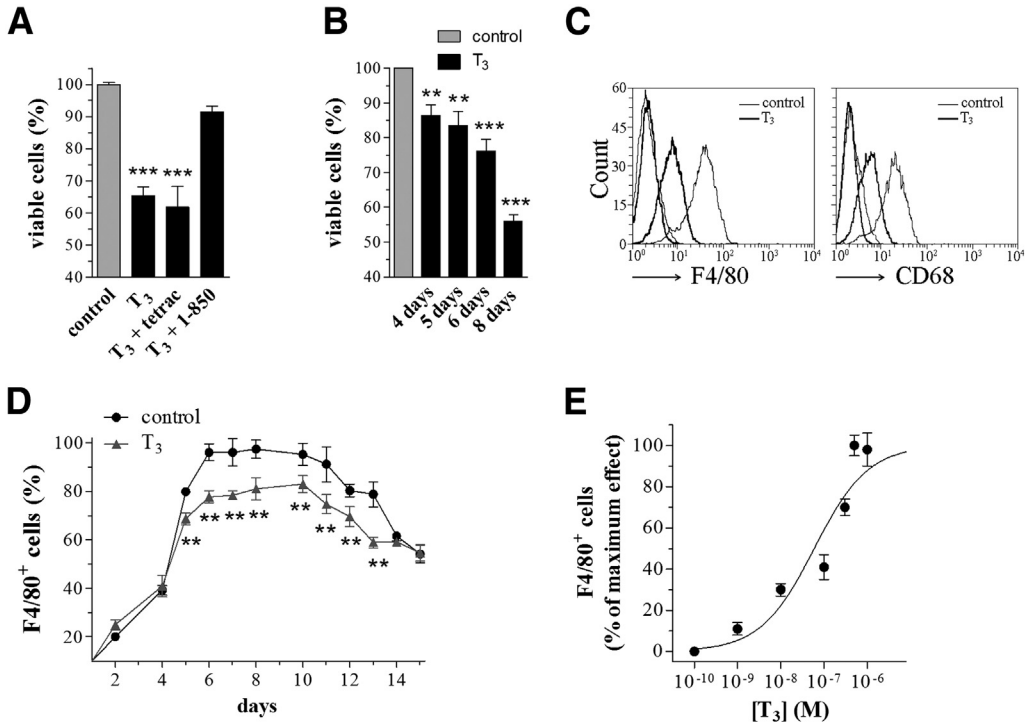

Figure 2 T_3 and differentiation of unpolarized macrophage cells. **A:** Bone marrow–derived cells were differentiated into unpolarized macrophages in the absence (control) or in the presence of 500 nmol/L T_3, 500 nmol/L tetrac, or 1 μmol/L 1-850. Cell viability was measured after 8 days of culture by counting vital cells (trypan blue exclusion test). Data are expressed by setting the control as 100%. Each histogram represents the means ± SEM of data from five independent experiments. **B–D:** Bone marrow–derived cells were differentiated into unpolarized macrophages in the absence (control) or in the presence of 500 nmol/L T_3. **B:** PI exclusion test (cell counting) by flow cytometry at different days of culture. Each histogram represents the means ± SEM of data from five independent experiments. Data are expressed by setting the control as 100%. **C:** Cell surface analysis by flow cytometry of F4/80 and CD68 expression after 8 days of culture. Results are representative of three to five independent experiments. **D:** F4/80-positive cells analyzed by flow cytometry at different days of culture. Each point represents the means ± SEM of data from five independent experiments. Data are expressed by setting as 100% the total adherent cells at each time point. **E:** Bone marrow–derived cells were differentiated into unpolarized macrophages in the presence of increasing concentrations of T_3. Concentration-response curve of T_3 effects on the number of F4/80$^+$ cells (by flow cytometry) at 8 days of culture. Each point represents the means ± SEM of data from three independent experiments. Data are expressed by setting as 100% the maximum effect of T_3. $^{**}P < 0.01$, $^{***}P < 0.001$ versus respective control.

all of the living cells that are adherent to the substrate had differentiated into macrophages. The fact that the levels of CD34 and CD11c markers were almost undetectable in both control and T_3-exposed cells (data not shown) demonstrates the virtual absence of hematopoietic- and dendritic-like phenotypes, although we cannot exclude that, in the presence of the hormone, progenitor cells may differentiate toward other lineages. Collectively, these data indicate that a bone marrow–derived cell suspension differentiates into a population of living cells expressing macrophage markers mostly between days 4 and 8, and that T_3 affects the ability of cells to attach and, thus, to completely differentiate to macrophages.

The concentration used in these *in vitro* experiments is higher than the T_3 serum concentration achieved in our *in vivo* models (Table 3); thus, we tested if the observed inhibitory effects of T_3 exposure on F4/80$^+$ cells were concentration dependent and if they are detectable at concentrations of T_3 comparable with the physiological serum concentration. As shown in Figure 2E, T_3 application caused a concentration-dependent effect on F4/80$^+$ cells (EC_{50} = 59 nmol/L). No significant effects were seen with concentrations <10 nmol/L, whereas the maximal changes were seen with concentrations >300 nmol/L.

Effects of T_3 on Differentiated Macrophage Cells

We investigated whether 500 nmol/L T_3 affected only unpolarized macrophage differentiation or acted also on differentiated macrophages. Cells were treated with T_3 for 24 hours after bone marrow–derived cell differentiation (8 days) onto macrophage cells. As shown in Figure 3A, in macrophage cells, the mRNA levels of TRα were not modified by T_3, whereas the hormone decreased the expression of TRβ1 mRNA. In addition, T_3 decreased the expression of the TRβ protein band, as confirmed by using Western blot analysis using an anti–TRβ-specific antibody (Figure 3B).

The number of adherent viable cells was not affected by T_3 application, as assessed by the PI exclusion test (Figure 3C). Remarkably, flow cytometry analysis revealed that T_3 increased the percentage of F4/80/CD14 (M1 marker)–positive cells by almost 50% (Figure 3D) and increased the levels of transcripts (Figure 3E) encoding for genes selectively expressed by M1-activated macrophages (as described in *Materials and Methods*). In addition, T_3 decreased the expression of genes selectively expressed by M2-activated macrophages, whereas the percentage of F4/80/CD206 (M2 marker)–positive cells remained undetectable (data not shown). Consistently, T_3 increased the release in the supernatant of the M1 cytokines, IFN-γ and TNF-α (Table 4). This effect was registered 48 hours after T_3 application, but not after 24 hours. Accordingly, in mouse macrophages *in vitro*, application of T_3 and T_4 at supraphysiological concentrations for 24 hours was found to be devoid of effects on cytokine release, including TNF-α.[20] Herein, we also show that the release of the M2 cytokine, IL-13, was not affected by T_3. Interestingly, T_3 also inhibited the migratory ability of macrophages by almost 37% (Figure 3F).

T_3 Regulates Macrophage Activation toward M1/M2

Macrophages were activated into polarized M1 or M2 via exposure to IFN-γ for 2 days or M-CSF + IL-4 for 4 days, respectively.[27,28] As detailed in *Materials and Methods*, the specificity of polarization was assessed by flow cytometry using various surface markers characterizing the various cell

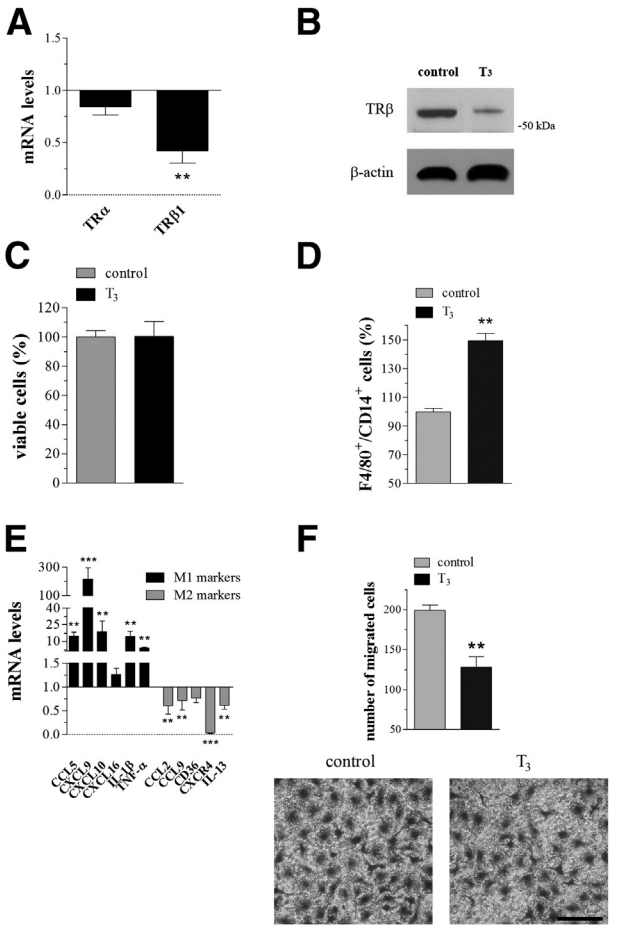

Figure 3 T_3 and differentiated macrophage cells. Macrophage cells were treated in the absence (control) or in the presence of 500 nmol/L T_3 for 24 hours. **A:** qPCR of TR mRNAs in the presence of T_3. Values are expressed as means ± SEM (n = 9) of the fold change over control (set as 1). **B:** Western blot analysis of TRβ protein (anti-TRβ *ab53170*). β-Actin (anti–β-actin *A5441*) was used as an internal standard. The image is representative of five independent experiments. pc, positive control. **C** and **D:** PI exclusion test (cell counting) and analysis of F4/80/CD14-positive cells, respectively, by flow cytometry. Each histogram represents the means ± SEM of data from 10 independent experiments. Data are expressed by setting the control as 100%. **E:** qPCR of mRNAs encoding for genes selectively expressed by M1 or M2 activated cells, in the presence of T_3. Values are expressed as means ± SEM (n = 4) of the fold change over control (set as 1). **F:** The number of migrated cells is plotted for each experimental condition (using 100 ng/mL M-CSF as chemoattractant) (**top panel**). Each histogram represents the means ± SEM of data from five independent experiments. Representative stains of migrated cells are depicted (**bottom panel**). Scale bar = 40 μm. $^{**}P < 0.01$, $^{***}P < 0.001$ versus respective control.

Table 4 Concentrations of Soluble Molecules in the Supernatants of Macrophages, as Assessed by ELISA

Name	At 24 hours	At 48 hours
IFN-γ		
Control	12.77 ± 1.03	14.20 ± 0.70
T_3	11.86 ± 0.81	28.26 ± 3.93*
IL-13		
Control	ND	ND
T_3	ND	ND
TNF-α		
Control	129.9 ± 11.20	176.3 ± 17.06
T_3	131.7 ± 11.38	293.2 ± 54.73*

Values are given in pg/mL × 10^6 cells.
*$P < 0.05$ versus respective control.
ND, not detectable.

subsets and gene expression profiles of specific patterns of cytokines and receptors. Then, the relative expression of TRs in unpolarized macrophage, M1, and M2 cells was investigated. As shown in Figure 4A, the transcript encoding TRα was similarly expressed by both unpolarized and polarized cells, whereas the expression of TRβ1 mRNA was enhanced in M2 cells (approximately 25 fold versus unpolarized macrophages) but not in M1. As depicted in Western blot experiments with anti-TRα/β antibody (Figure 4B), we detected only the TRβ-specific band in M1 and M2 cells, which appeared to be up-regulated in M2 when compared with unpolarized macrophage cells. In addition, immunofluorescence analysis with anti-TRα1 and anti-TRβ1 antibodies, in combination with the anti-F4/80 antibody and Hoechst staining, showed the absence of TRα1-specific signal in M1 (Figure 5A) and M2 (Figure 5B) cells. In contrast, strong perimembranous and cytoplasmic/perinuclear labeling for TRβ1 was found in both cell models. The nuclear staining for TRβ1 was generally scarce and diffuse.

In another set of experiments, T_3 was administered at 500 nmol/L to unpolarized macrophages daily, starting from the beginning of the activation process. When combined with M1 stimulation, T_3 significantly increased the number of F4/80$^+$ also positive for CD14, while not affecting the overall percentage of F4/80-positive cells (Figure 6A). T_3 exposure differentially modulated the expression of M1 marker genes. In particular, it increased the expression of CXCL16 and TNF-α mRNAs, whereas, unexpectedly, it decreased the levels of transcripts encoding CCL5 and IL-1β (Figure 6C). When combined with M2 stimulation, T_3 significantly decreased the number of F4/80$^+$ also positive for CD206, while not affecting the overall percentage of F4/80-positive cells (Figure 6B). Consistently, T_3 decreased the expression of the M2 marker genes, *CCL2*, *CCL9*, *CD36*, and *IL-13* (Figure 6C).

Arginase 1, a manganese metalloenzyme that catalyzes the hydrolysis of L-Arg to L-ornithine and urea, is one of the most specific markers of M2 macrophages.[68–70] M2 activation led to a marked up-regulation of arginase 1 that was inhibited by treatment with 500 nmol/L T_3 (Figure 7A).

We finally assessed the effect of 500 nmol/L T_3 at the functional level. To this end, we relied on phagocytosis, which is increased in M1 cells compared with unpolarized or M2 macrophages.[28] Phagocytosis was measured using latex beads coated with fluorescently labeled IgG, by determining, in flow cytometry analyses, the percentage of positive (ie, actively phagocytosing) cells and the extent of phagocytosis as MFI. As shown in Figure 7B, phagocytic M2 cells were 28% less than M1 cells and the extent of phagocytosis in M1 cells was 3.9-fold higher than that of M2 cells. Of interest, T_3 increased both the percentage of M1 cells participating in phagocytosis and the extent of phagocytosis (Figure 7C).

T_3 Affects Macrophage Subsets *in Vivo*

We investigated whether the sensitivity of macrophages to T_3, determined in *in vitro* experiments, was observed *in vivo*. Immunofluorescence analysis with anti-TRα1 and anti-TRβ1 antibodies, in combination with the anti-F4/80 antibody and Hoechst staining, showed the substantial

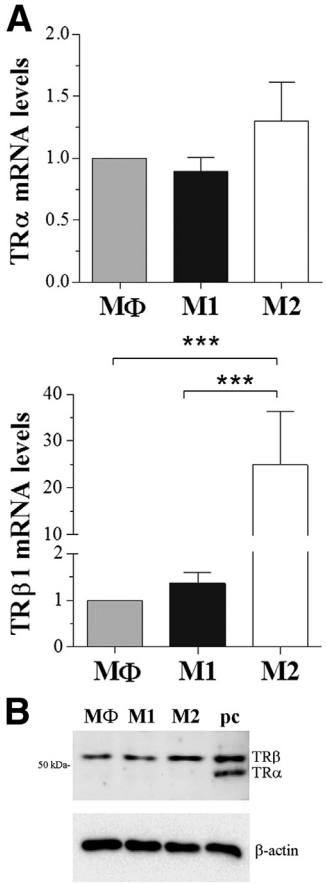

Figure 4 TR expression in unpolarized macrophages (MΦ), M1, and M2 cells. **A:** qPCR of mRNAs encoding for TRα and TRβ1 genes. Values are expressed as means ± SEM ($n = 3$ to 5) of the fold change over unpolarized macrophages (set as 1). ***$P < 0.001$. **B:** Western blot analysis performed with the anti-TRα/β (*sc-772*) antibody. β-Actin (anti–β-actin *A5441*) was used as internal standard. The image is representative of four independent experiments. pc, positive control.

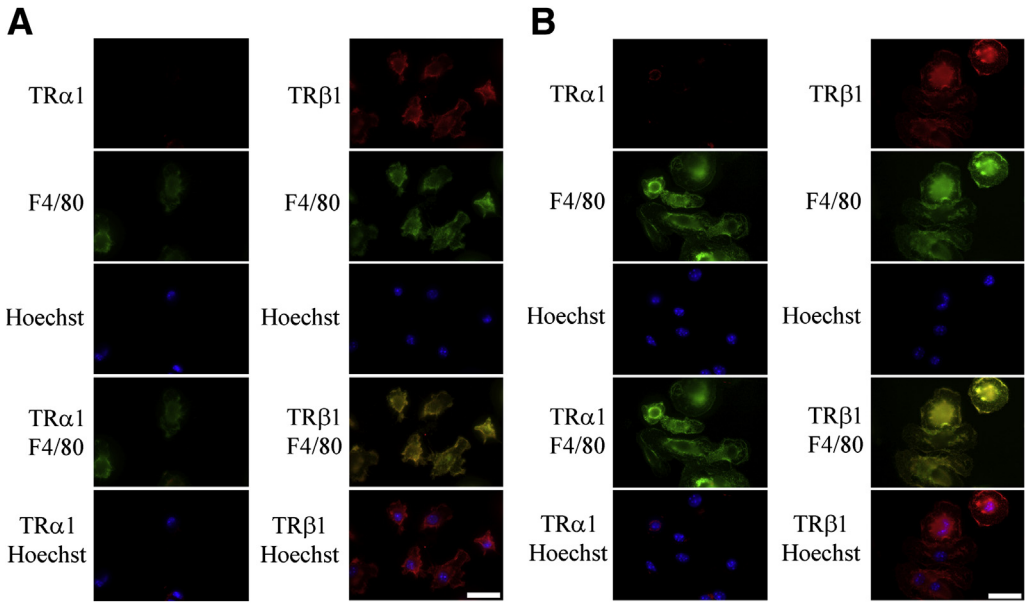

Figure 5 Immunofluorescence analysis of TR expression in M1 (**A**) and M2 (**B**) cells stained with anti-TRα1 (*sc-10819*) and anti-TRβ1 (*sc-738*) antibodies (red). M1 and M2 cells were also stained with anti-F4/80 antibody (green) and with Hoechst dye (blue). The images are representative of three independent experiments. Scale bars: 20 μm.

absence of TRα1-specific signal in macrophages isolated from the peritoneal cavity (Figure 8). In contrast, we found strong perimembranous and cytoplasmic/perinuclear labeling for TRβ1, whereas the nuclear staining was generally scarce and diffuse.

Then, we took advantage of an established model of hypothyroid mice (Table 3) that was compared with euthyroid mice. As shown in Figure 9A, quantitative image analysis, by flow cytometry, of isolated peritoneal cavity cells revealed similar levels of cells that positively immunostained for F4/80 in both euthyroid and hypothyroid mice; a small, but not significant, decrease of F4/80^{+} cells was obtained after i.p. injection of hypothyroid animals with 0.2 μg/g body weight per day T_3 for 5 days. These results suggest that T_3 levels do not perturb drastically the total content of peritoneal macrophages. Two coexisting peritoneal macrophage subsets have been recently distinguished in mice, with unique phenotypic and functional characteristics.[56,71] The ones expressing high levels of F4/80 (F4/80^{high}) were defined as large peritoneal macrophages (LPMs), and the subset of remaining macrophages expressing low levels of F4/80 (F4/80^{low}) was defined as small peritoneal macrophages (SPMs). By using the gating strategy reported in Figure 9B, we identified such different subsets of macrophages (SPM, 10.9% ± 2.5%; and LPM, 42.0% ± 3.8% versus total cell number; $n = 8$). In hypothyroid mice, the SPM population became the dominant macrophage subset (41.2% ± 5.9%; $n = 10$; $P < 0.0001$ versus euthyroid) and LPM frequencies decreased (11.9% ± 1.7%; $n = 10$; $P < 0.0001$ versus euthyroid). At 5 days, 0.2 μg/g body weight per day T_3 i.p. injection prevented the effect of hypothyroidism on the SPM subset (12.1% ± 0.4%; $n = 5$; $P < 0.0001$ versus hypothyroid), and a slight, but significant, effect was achieved on LPM frequencies (19.6% ± 2.9%; $n = 5$; $P < 0.05$ versus hypothyroid). In both euthyroid and hypothyroid animals, we also found that a few (<8%) of the F4/80-positive cells expressed CD14 or CD206 (data not shown).

TH Levels Affect Animal Mortality and Peritoneal Macrophage Subsets during Endotoxemia

LPM and SPM differ markedly in their *in vivo* responses to inflammatory stimuli,[56] and M1 cells (CD14^{+}) have a classic proinflammatory profile; we evaluated whether alterations of TH levels affected the animal survival rate during systemic inflammation. To this end, we injected euthyroid and hypothyroid mice i.p. with LPS to induce endotoxemia, and we recorded the mortality of the animals over 96 hours. As shown in the Kaplan-Meier curve of Figure 10A, hypothyroid mice exhibited significantly increased mortality during the course of the experiment (10% survival of hypothyroid mice versus 35% survival of euthyroid mice). When injected i.p. with 0.2 μg/g body weight per day T_3 for 5 days before the onset of endotoxemia, hypothyroid animals were significantly protected from death (70% survival). To verify the involvement of TRs, hypothyroid mice were also injected i.p. with 20 μg/g body weight per day AMIO for 2 weeks before the treatment with T_3 and the onset of endotoxemia. AMIO is an antiarrythmic drug that acts *in vitro* and *in vivo* as a TRα1 and TRβ1 antagonist via its major metabolite, desethylamiodarone.[55] It has been recently reported that mice injected i.p. with 80 μg/g body weight per day AMIO for 25 days had only mild alterations in TH economy, with doubling of plasma TSH concentrations in the face of steady plasma T_4 and T_3 levels.[51] As shown in Figure 10A, the

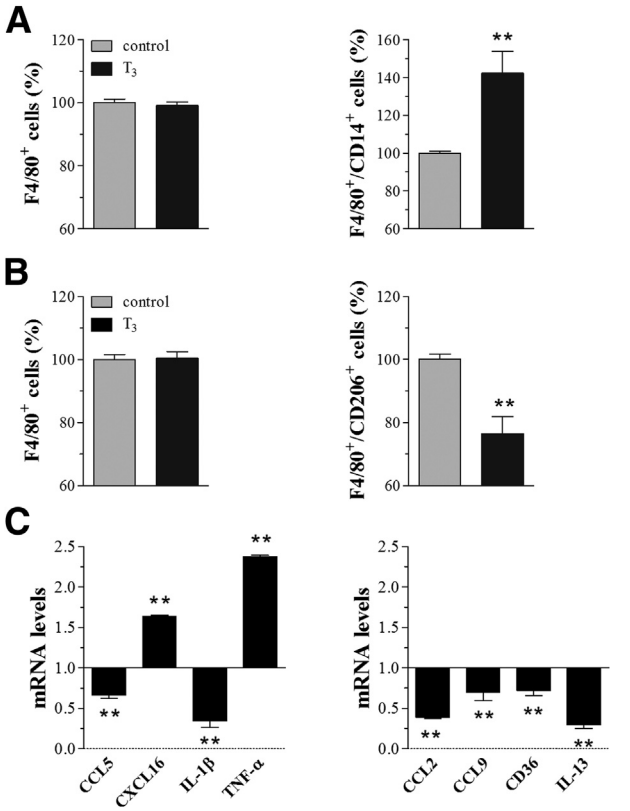

Figure 6 T_3 and activation of M1/M2 cells. Macrophages were polarized into M1 or M2 in the absence (control) or in the presence of 500 nmol/L T_3. **A** and **B**: Flow cytometry analysis of F4/80-positive cells and F4/80/CD14- or F4/80/CD206-positive cells in M1- and M2-stimulating conditions, respectively. Each histogram represents the means ± SEM of data from four to six independent experiments. Data are expressed by setting the control as 100%. **C**: qPCR of mRNAs encoding for M1 or M2 genes in M1- or M2-stimulating conditions (**left** and **right panels**, respectively), and in the presence of T_3. Values are expressed as means ± SEM ($n = 4$) of the fold change over control (set as 1). $^{**}P < 0.01$ versus respective control.

protective effects of T_3 in our system were decreased in AMIO-injected mice (45% survival).

We next delineated TH/macrophage phenotype interactions during systemic inflammation, using euthyroid and hypothyroid mice. Animals were treated i.p. with LPS for 16 hours (ie, a time interval before animal death). As previously suggested in a model of mouse injury obtained by cecal ligation and puncture,[72] it is likely during this period that inflammatory changes differentiate between mice that live and die. At the end of the experiment, the phenotype of peritoneal F4/80$^+$ cells was determined. In euthyroid animals, a marked shift in the SPM/LPM ratio (SPM, 37.8% ± 3.9%; LPM, 15.0% ± 1.7%; $n = 4$; $P < 0.001$ versus untreated euthyroid) was observed after LPS i.p. injection (Figure 10B). It was previously demonstrated that significantly lower doses of LPS (10 μg versus 10 to 15 μg/g body weight, as in our experiments) did not change mouse SPM and LPM frequencies at 20 hours after i.p. LPS injection, whereas by 2 days after stimulation, SPM becomes the highly dominant subset.[56] Taken together, these results indicate a correlation between LPS doses and time-response of macrophage phenotype. As shown in Figure 10B, we also found that hypothyroid mice showed an additional increase of the SPM frequencies (58.6% ± 3.3%; $n = 3$; $P < 0.05$ versus LPS-injected euthyroid mice), whereas LPM frequencies did not substantially change (19.82% ± 2.7%) compared with LPS-injected euthyroid mice. In contrast, hypothyroid mice i.p. injected for 5 days with 0.2 μg/g body weight per day T_3 and treated with LPS showed a dramatic decrease of the SPM/LPM ratio (SPM, 27.4% ± 6.7%; LPM, 35.2% ± 4.5%; $n = 3$; $P < 0.05$ versus both LPS-injected euthyroid and hypothyroid mice). After LPS injections, F480$^+$/CD14$^+$ expression in SPM or LPM cells did not change in euthyroid, hypothyroid, or hypothyroid mice treated with T_3 (Figure 10C), whereas the presence of F4/80-positive cells expressing CD206 was almost undetectable (data not shown).

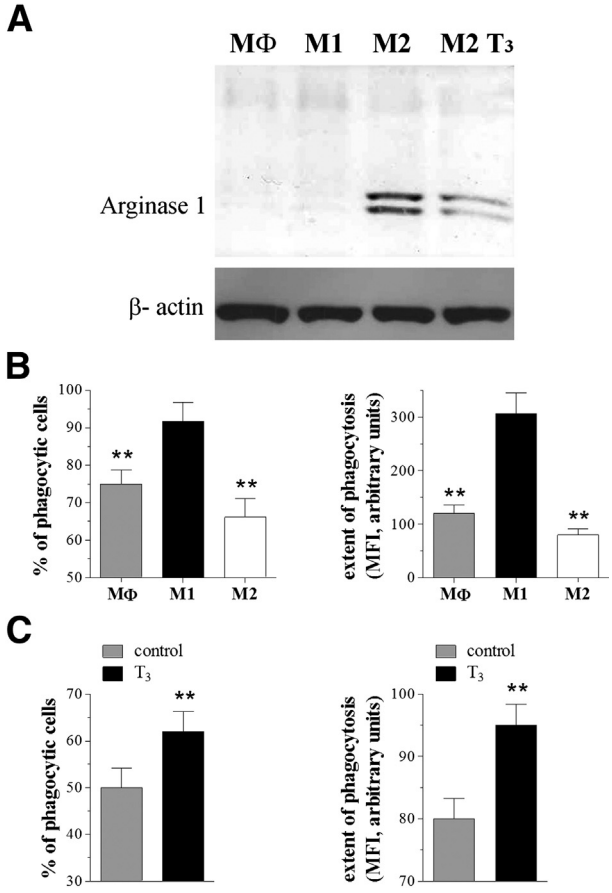

Figure 7 **A**: Western blot analysis of arginase 1 (anti—arginase 1 *sc-20150*) in unpolarized macrophage, M1, and M2 cells and in the M2-stimulating condition in the presence of 500 nmol/L T_3. β-Actin (anti—β-actin *A5441*) was used as internal standard. The image is representative of three independent experiments. **B**: Percentage of phagocytic cells and MFI in unpolarized macrophage, M1, and M2 cells after 3 hours of incubation with latex beads coated with fluorescently labeled IgG. Each histogram represents the means ± SEM of data from five independent experiments. **C**: Percentage of phagocytic cells and MFI in M1-stimulating conditions and in the presence of 500 nmol/L T_3, at 1 hour of bead exposure. Each histogram represents the means ± SEM of data from five independent experiments. $^{**}P < 0.01$ versus M1 or respective control.

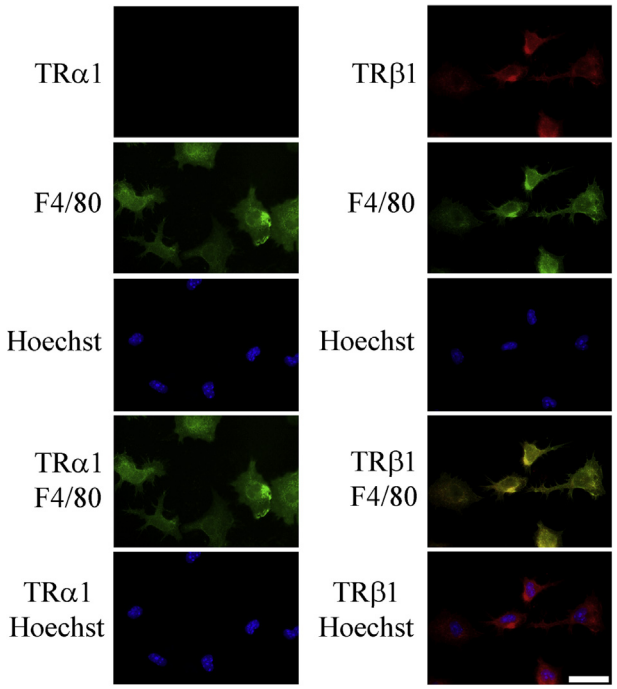

Figure 8 Immunofluorescence analysis of TR expression in peritoneal macrophages stained with anti-TRα1 (*sc-10819*) and anti-TRβ1 (*sc-738*) antibodies (red). Macrophages were also stained with anti-F4/80 antibody (green) and with Hoechst dye (blue). The images are representative of three independent experiments. Scale bar = 20 μm.

Discussion

This study identifies a homeostatic link between THs and the pathophysiological role of macrophages. Macrophages fulfill key functions in immunity, and these properties may change in response to the variable microenvironmental signals of the local milieu.[13,14,22] There is evidence that T_3 drives maturation and signaling of dendritic cells,[11,12] the cells most closely associated with macrophages.[13] We show that T_3 affects macrophage phenotype and function, thus extending the role of this hormone as an immune-regulating factor.

In Vitro Results

Bone marrow–derived monocytes enter peripheral blood and circulate for several days in an inert state before entering tissues and differentiating into tissue-resident macrophages. The endocrine system participates in regulating their differentiation and maturation.[5,73,74] Our results indicate a negative role of T_3 in triggering the differentiation of monocytes into macrophages in culture. Physiological concentrations of T_3 were suggested to regulate the cell population growth of human hematopoietic cells.[58] Compared with the serum concentration of T_3 measured in euthyroid mice[50–52] (present results), and disregarding any degradation during the culture time, the T_3 concentration used under our experimental condition *in vitro* (500 nmol/L) is higher than the physiological range. In mouse macrophages *in vitro*, T_3 was recently shown to be active at supraphysiological concentrations (100 nmol/L).[20] In addition, tissues derive most of their T_3 from local de-iodination of T_4 and cells can regulate their T_3 content by means of active transport.[7,8] Thus, defining the exact concentration of active T_3 acting locally on tissue cells versus the one measured in the serum is difficult. In addition, although cell cultures mimic the *in vivo* systems, the more vigorous metabolism *in vitro* can degrade T_3 rapidly, dramatically reducing the initial dose after 24 hours, as described in primary cultures of rat brain cells.[75] Consistently, a wide range of hormone concentrations has been used *in vitro* to define the actions of THs. Several reports describe T_3 effects at subnanomolar-nanomolar concentrations in cell cultures,[11,12,21,58,65,76–79] whereas others used T_3 concentration of two to three orders of magnitude higher than the one measured in the serum.[30,47,64,80–84] As previously shown, the binding affinity curves of T_3 at TRs *in vitro* indicate that the maximal receptor occupancy occurs at a higher than physiological hormone concentration (ie, >10 nmol/L).[62] We demonstrate herein that concentrations of T_3 comparable with free serum concentration were not sufficient to elicit detectable effects in *in vitro* primary cultures of monocytes/macrophages, and maximal T_3 effects were achieved only at higher levels, as already reported in other systems, including macrophages.[47,80,84]

An important conclusion of our study is the demonstration that T_3 induces unpolarized macrophage cells to display a classically activated (M1) signature, as revealed by the expression analysis of surface proteins, gene markers, and cytokine release. Chemotaxis represents an important function by which macrophages confront certain pathogens. The motility *in vitro* of mouse M1 cells was reported to be lower than that of M2 cells[28]; our observation that T_3 induced inhibition of unpolarized macrophage migratory ability is consistent with an M1-priming effect of T_3. This is also observed on activated, polarized macrophages. Indeed, we show that T_3 reversed M2 activation while enhancing that of M1 cells, even at a functional level. Macrophages are strategically located throughout the body tissues, where they ingest and process foreign materials, dead cells, and debris.[85] Our results indicating a positive fine-tuning role for T_3 in promoting the phagocytic activity of M1 cells are consistent with evidence that phagocytosis is increased in M1 cells compared with M2 macrophages[28] (present results) and with earlier observations in mice.[19] Different results have been reported in hypothyroid rats.[16] In particular, incubation *in vitro* of mouse peritoneal macrophages with higher than physiological concentrations of T_3 induced no modifications in the phagocytic capacity, whereas phagocytosis was stimulated by physiological concentrations of T_4.[17] In a recent study, high concentrations of T_3 and T_4 stimulated bacterial phagocytosis of mouse macrophages.[20] Accordingly, *in vivo* T_4 treatment of mice induced a slight increase in peritoneal macrophage phagocytosis.[18] As previously suggested,[6] it is possible that the different responses

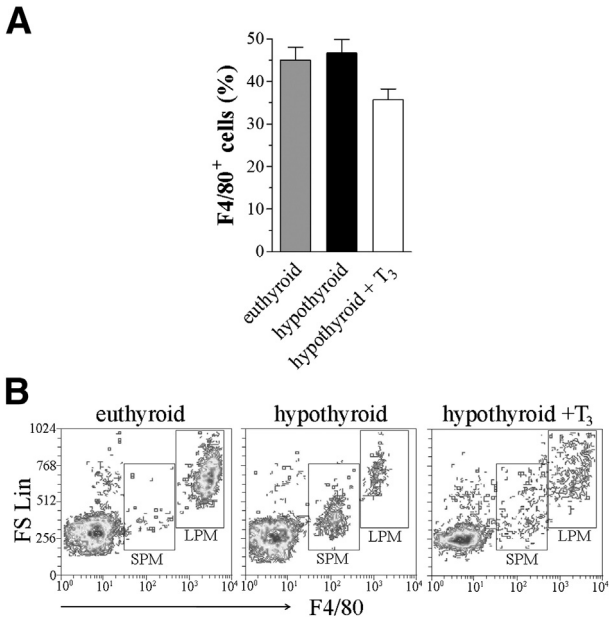

Figure 9 T_3 and *in vivo* content of murine peritoneal macrophages. Cell surface analysis by flow cytometry of isolated peritoneal cavity cells in euthyroid and hypothyroid mice, as well as in hypothyroid mice injected i.p. for 5 days with 0.2 μg/g body weight per day T_3. **A:** Analysis of F4/80-positive cells. Data are expressed by setting as 100% the total cell number ($n = 8$ to 18). **B:** Gating strategy used to identify the macrophage subsets of large cells expressing high levels of F4/80 (LPM) and small cells expressing low levels of F4/80 (SPM). FSLin, forward scatter in linear mode (cell size) ($n = 5$ to 10).

elicited by THs depend on the type of inert particles phagocytosed. In this respect, in our experiments, we assayed the engulfment of IgG-labeled particles; however, the effects of THs on other forms of phagocytosis (eg, the noninflammatory complement-mediated phagocytosis) would deserve further systematic investigation. It is generally assumed that M1 or M2 activation *in vivo* represents extremes of a continuum in a universe of activation states and mixed phenotypes, and coexistence of cells in different activation states has been observed in preclinical/clinical conditions.[22] Our data reporting the inhibition of leading M1 gene markers, such as IL-1β or CCL5, after T_3 exposure revealed a somehow mixed phenotype. The switching induced by T_3 in activated cells appears, therefore, complex and represents an important biological issue that needs to be investigated further.

The use of T_3 antagonists in our system indicated that T_3 effects are initiated by intracellular TRs. Among TRs, we found that bone marrow–derived unpolarized macrophage, M1, and M2 cells expressed detectable levels of transcripts encoding for TRα and TRβ1, the latter being positively regulated during M2 activation. The mRNAs for TRα and TRβ were previously detected in bone marrow–derived macrophages and found to increase in IFN-γ–primed cells.[26] However, when considering these results, mRNA expression may correlate poorly with levels of TR protein. Our results indicate that unpolarized macrophage, M1, and M2 cells expressed TRβ1 protein at the intracellular level, whereas TRα1 protein was undetectable. In agreement with mRNA data, M2 expressed higher levels of TRβ. At the mRNA and/or protein level, the presence of TRα1 and TRβ1 was reported in rat skeletal mast cells and human bone marrow $CD34^+$ cells,[58,61] as well as at different stages of mouse dendritic cell maturation.[11,12]

We show the following in bone marrow–derived macrophages: i) only TRβ1 is expressed in differentiated unpolarized macrophages and M1/M2 activated cells, ii) T_3 in differentiated macrophages significantly decreases the expression of TRβ1, and iii) TRβ1 is strongly up-regulated in M2 cells. Collectively, these data suggest a major functional role of TRβ1 in mediating T_3 effects, including the

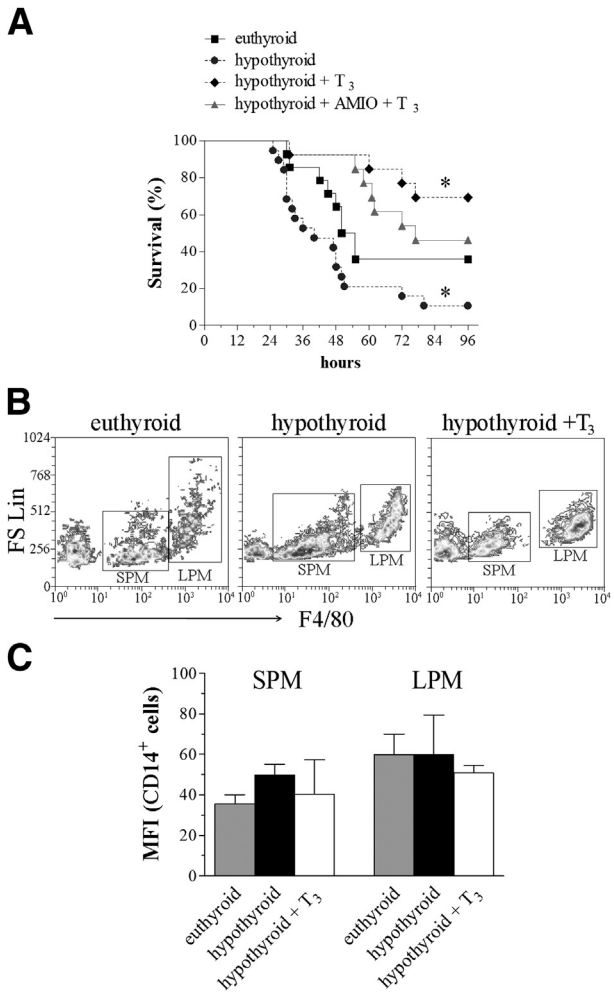

Figure 10 T_3 and survival in murine endotoxemia induced by i.p. injection with LPS. **A:** Percentage survival (Kaplan-Meier curve, $n = 13$ to 18) of euthyroid and hypothyroid mice. Hypothyroid mice were also injected i.p. with 0.2 μg/g body weight per day T_3 alone or with 20 μg/g body weight per day of AMIO for 5 days before the onset of endotoxemia. $^*P < 0.05$ versus euthyroid value. **B** and **C:** Cell surface analysis by flow cytometry of isolated peritoneal cavity cells in euthyroid and hypothyroid mice and in hypothyroid mice injected i.p. for 5 days with 0.2 μg/g body weight per day T_3. Mice were injected with LPS for 16 hours. **B:** Gating strategy used to identify the macrophage subsets of large cells expressing high levels of F4/80 (LPM) and small cells expressing low levels of F4/80 (SPM). FSLin, forward scatter in linear mode (cell size) ($n = 3$ to 4). **C:** Analysis of CD14 expression (MFI ± SEM) on LPM and SPM $F4/80^+$ cells ($n = 3$ to 4).

promotion of unpolarized macrophage cells toward the M1 versus M2 signature. This issue, however, deserves further investigations. Of interest, TRβ1 was shown to mediate the T_3-induced maturation and signaling of mouse dendritic cells.[11,12]

In Vivo Results

Given the effects of T_3 on unpolarized macrophage maturation *in vitro*, we investigated the effects of THs in the modulation of macrophage phenotype/recruitment *in vivo*. Although macrophages were historically considered to be derived from the blood monocyte reservoir, numerous studies have since demonstrated that, under steady-state conditions, resident tissue macrophage populations are largely maintained through local proliferation.[86] Inflammatory insults, however, result in the rapid recruitment of blood-borne precursors to the respective tissue macrophage compartment.[86] Our data demonstrate that circulating T_3 increased the content of LPMs, the resident macrophages in the peritoneal cavity,[56] while reducing the content of SPMs, the recruited monocyte-derived cells.[56] The half-life of macrophages in tissues varies from a few days up to several months. Thus, the decreased content of LPM cells in hypothyroid mice may account, at least in part, on the effect of T_3 levels on macrophages that were in the peritoneal cavity at the beginning of the hypothyroid model and were still there when the analysis was done.

Tissue macrophages, and newly recruited monocytes, ensure baseline tissue homeostasis and prevent constant inflammation.[13,22] Classic inflammatory stimuli, such as microbial insult (ie, LPS), frequently occur with concomitant type 2 helper T cell (Th2) cytokine (ie, IL-4) production. It has been recently shown that both resident and recruited (ie, LPM and SPM, respectively, as identified in our model) mouse macrophages can be activated and driven to proliferate by a Th2 environment *in vivo*.[71] It was demonstrated that a Th2 environment was sufficient to drive accumulation of tissue macrophages through self-renewal, indicating that the mechanism of local expansion in type 2 inflammation does not require enhanced bone marrow activity and may deliberately avoid inflammatory cell recruitment and the associated potential for tissue damage.[71] Thus, the proliferation *in situ* has been proposed as an alternative mechanism of inflammation that allows macrophages to accumulate in sufficient numbers to perform critical functions in the absence of potentially damaging cell recruitment.[71] In light of these observations, it is tempting to speculate that T_3 contributes to limit inflammation by promoting the proliferation of macrophages *in situ*, while inhibiting the potentially damaging cell recruitment from monocyte cell pools. Accordingly, we show that circulating T_3 significantly protected mice against inflammation induced by LPS i.p. injection, a model of endotoxemia resembling sepsis.[45,57] This is consistent with previous observations that high circulating levels of THs oppose proinflammatory mechanisms in which monocytes and macrophages are involved, although discrepancies on TH effects have been also reported.[6] Our findings are in good agreement with published reports on the beneficial role of THs in mice with severe meningococcal sepsis symptoms[20] or in rats during severe sepsis induced by cecal ligation and puncture.[87,88] Relevant data on TH levels as indicators of sepsis severity and predictors of mortality come from human studies. For instance, in patients with septic shock, tissue responses are orientated to decrease production and increase degradation (muscle) or decrease uptake (adipose tissue) of T_3, as well as to decrease thyroid hormone actions.[89] In addition, T_3 levels were significantly lower in patients with sepsis compared with patients with an inflammatory response without underlying infection,[90] and the levels of THs have been found to be a parameter for evaluating meningococcal septic shock severity.[91,92] A systematic review regarding the association between thyroid hormone abnormalities and the outcome of patients with sepsis or septic shock suggests the existence of an association between lower T_3 or T_4 and worse outcome, although definite conclusions on the described issue cannot be drawn on the basis of the data available.[93] In this respect, because thyroid hormone abnormalities are common in septic patients, future studies should aim to more clearly establish the strength of the previously mentioned association and to determine whether TH supplementation could be beneficial for the outcome of septic patients.

The functional plasticity of macrophages in the course of inflammation and its resolution serve as a means of regulating inflammation in space and time,[94] and sepsis is a case of deregulated inflammation.[95] Although the mechanisms by which T_3 protects mice against LPS-induced endotoxemia are unclear, the use of hypothyroid mice provides evidence that decreased T_3 levels increased SPM frequencies induced by LPS i.p. injection; however, the restoration of T_3 levels decreases SPM and increases LPM frequencies. This suggests that the anti-inflammatory effect of the T_3 system is coupled to the functional modulation of SPM (potentially damaging recruited from bone marrow–derived cells) and LPM (potentially beneficial resident) subsets.[56,71] This is consistent with our *in vitro* results showing a negative role of T_3 in triggering the differentiation of macrophages from bone marrow–derived cells. The fact that peritoneal macrophages expressed TRβ1 protein, whereas TRα1 detection failed, and that the TRα1/β1 antagonist, AMIO, inhibited T_3-induced protection against LPS-induced endotoxemia strongly suggest TRβ1 as the major player mediating T_3 effects *in vivo*, in agreement with the *in vitro* results. THs have been recently suggested to control meningococcal septicemia in mice by up-regulating the macrophage production of nitric oxide,[20] a well-established marker of the M1 phenotype.[13,22,28] In this regard, *in vivo* stimulation would classify SPMs and LPMs as M1 cells because both produce large amounts of nitric oxide in response to LPS.[56] Thus, the fact that the M1/M2 framework does not readily accommodate the SPM and LPM subsets should be considered. Our *in vivo* data suggest

that T_3 protection against LPS-induced endotoxemia is caused more by changes in macrophage migration than the differential expression of M1 markers. Accordingly, during the early phase of inflammation, M1 macrophages mediate microbicidal activity, recruitment, and activation of adaptive immune cells and trigger a full-fledged inflammatory response, whereas, during resolution, these cells switch to an anti-inflammatory and tissue remodeling mode.[94]

Conclusions

The balance of a positive action of T_3 in inflammation versus a potential detrimental one because of the novel link between THs and macrophage maturation we demonstrate opens new perspectives on the interactions between the endocrine and immune systems. The key role of T_3 in inhibiting the differentiation of monocytes into macrophages, while favoring the M1 activation, indicates that this hormone has significant functions in the regulation of immune responses. The increase of resident versus monocyte-recruited macrophages may be an important effector of T_3-induced protection against the systemic inflammatory response of endotoxemia, which may be developed in therapeutic strategies to counteract pathological conditions disregulating, at least in part, TH homeostasis.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2013.10.006*.

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