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Inhibition of the Growth of *Toxoplasma gondii* in Immature Human Dendritic Cells Is Dependent on the Expression of TNF-α Receptor 2

Anja Giese,*† Silke Stuhlsatz,* Walter Däubener,* and Colin R. MacKenzie**

An effective immunity to *Toxoplasma gondii* in humans is dependent on the cellular immune response. *Toxoplasma* can infect and replicate in almost all nucleated cells, and the most important cytokine regulating the growth in humans is IFN-γ; however, the role of TNF-α has to date been largely described to be synergistic. We show that, compared with mature human dendritic cells (mDC), immature human DC (iDC) demonstrate a reduced parasite proliferation when infected with *Toxoplasma*. This toxoplasmostasis was only present in iDC after 11 days of culture and was not present in DC that had been matured ex vivo using a cytokine mixture (mDC). Spontaneous toxoplasmostatic activity has previously only been described in fresh human monocytes, and the mechanism involved is as yet unclear. We show that, in comparison with an absence of expression in mDC, TNF-R2 is expressed in both iDC and monocytes infected with *Toxoplasma*, and furthermore, that blocking the TNF-R2 with Abs abrogates the toxoplasmostasis in the iDC. These findings demonstrate a functional role for TNF-R2 in the newly described spontaneous toxoplasmostasis of iDC.


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The obligate intracellular protozoan parasite *Toxoplasma gondii* causes a chronic asymptomatic infection in immunocompetent individuals. The ability to evade the immune system over decades and then, in the event of severe immunosuppression, to reappear in the form of a life-threatening encephalitis makes *Toxoplasma* both an important health hazard as well as an interesting organism to study the survival techniques of intracellular organisms. Furthermore, *Toxoplasma* is capable of replicating in almost every nucleated cell, and the control of growth and suppression of reactivation require IFN-γ (1). A number of mechanisms have been described to be effective in controlling *Toxoplasma* infection, such as NO (2), oxygen radical production (3), depletion of iron (4), and depletion of tryptophan (5). The mechanism involved in human macrophages, however, remains unclear. Whereas NO as a defense mechanism against *Toxoplasma* in human cells is controversial (6–8) and tryptophan depletion in human macrophages is not responsible for toxoplasmostasis (9), reactive oxygen intermediates appear to be at least partly responsible for an anti-*Toxoplasma* defense (10). Recently, toxoplasmostatic activity was shown in murine dendritic cells (DC),3 and this was described to be neither due to NO nor tryptophan depletion, but to be abrogated by coincubation with oxygen radical scavengers (11).

DC have typically been ascribed an immune stimulatory and regulatory function and not necessarily an effector function. Furthermore, cells of the mononuclear line have been proposed to play a part in the dissemination of *Toxoplasma* by offering the parasites asylum from the humoral and cellular immune systems, and moreover, by acting as couriers from the infection site to distant organs where encystation takes place (12). Recently, it has been reported that human monocytes and DC are permissive for *Toxoplasma* proliferation, which would support this hypothesis (13). In direct contrast to this, fresh human monocytes display a spontaneous *Toxoplasma* growth-inhibitory effect (14).

In murine toxoplasmosis, the IFN-γ-mediated effect is dependent on TNF-α (15), which exerts its effect exclusively via TNF-R1 and not via TNF-R2 (16, 17). In humans, the role of TNF receptors is less well described. A recent report described an upregulation of TNF-R2 and inhibition of production of TNF-α in the human monocyte cell line THP-1 after infection with *Toxoplasma* (18). TNF-α has its major effect through the TNF-R1, and it has been proposed that TNF-R2, due to its higher affinity for TNF-α, serves to capture TNF-α and pass it on to TNF-R1, which is then involved in signal transduction (19). Signal transduction via TNF-R2 has not been described in human cells.

Our aim in this study was to elucidate the underlying mechanism or mechanisms for the observed resistance of immature DC (iDC) to *Toxoplasma* growth. We set out to determine whether the innate toxoplasmostatic activity of these iDC was due to a known characteristic of iDC such as apoptosis or IFN-γ secretion, or perhaps due to some other cytokine-induced mechanism. The cytokine gene expression of iDC, mature DC (mDC), and monocytes was thus examined, as well as the permissiveness of the DC.

In the present study, we provide evidence that, in addition to fresh human monocytes, human DC with an immature phenotype (iDC) display a spontaneous toxoplasmostasis. To our knowledge, this is the first report of such activity in cells other than monocytes. Furthermore, this activity was restricted to cells that had been in culture for ~11 days, which may suggest a selection of a subpopulation of DC exhibiting this effect. Compared with iDC, DC that were matured ex vivo using the cytokine mixture IL-1, IL-6, TNF-α, and PGE2 (mDC) displayed no inhibition of *Toxoplasma* growth without IFN-γ stimulation. Both iDC and mDC showed a
strong Toxoplasma growth inhibition after stimulation with IFN-γ. Our experiments demonstrate that in both iDC and monocytes that exhibit this toxoplasmostasis, infection with Toxoplasma results in the expression of TNF-R2. In comparison, mDC and macrophages do not express TNF-R2 after infection. Furthermore, by blocking the TNF-R2 using neutralizing anti-TNF-R2 Abs, we demonstrate that the TNF-R2 has a functional role in the toxoplasmostasis of iDC. We know of no other report showing an association between TNF-R2 and an effector function in DC.

Materials and Methods

Differentiation and culture of monocyte-derived DC

PBMC were isolated fromuffy coats ofheparinized blood of healthy volunteer donors at the blood bank of the University Hospital (Düsseldorf, Germany) by Ficoll Hypaque (Amersham Biosciences, Freiburg, Germany) density centrifugation. CD14+ cells were separated using immunomagnetic bead selection (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. The obtained monocytes were shown in FACS analysis to be >95% pure, and subsequent (5 × 10⁴) cells were cultured in 3 ml of IMDM supplemented with 10% FCS (BioWhittaker, Apen, Germany), 75 ng/ml GM-CSF (Novartis Pharmaceuticals, Kundl, Austria), and 100 U/ml IL-4 (R&D Systems, Wiesbaden, Germany). Cytokines were replenished every 3 days by removing 1 ml of supernatant and replacing it with fresh medium containing the same final concentration of cytokines. After a maximum incubation period of 10 days, cells were harvested and split into two subcultures. To obtain mDC, one population was supplemented with additional IL-1 (1000 U/ml), TNF-α (1000 U/ml) (both R&D Systems), IL-6 (1000 U/ml; PeproTech, Rocky Hill, NJ), and PGE2 (1 µg/ml; Sigma-Aldrich, St. Louis, MO) for an additional 3 days. A fraction of the DC was submitted to FACS analysis to confirm their maturation status.

Flow cytometry

DC were stained using FITC-conjugated CD86, HLA-DR, and CD80 mAbs and PE-conjugated CD83, CD40, and CD11b mAbs (BD Biosciences, San Jose, CA). To block Fc receptors, cells were first incubated for 20 min in 1% rabbit serum in PBS, pH 7.3 (Serotec, Serag, Wiessen, Germany) with 0.1% sodium azide (Sigma-Aldrich) and washed with PBS containing 5% FCS and 0.1% sodium azide. The subsequent staining was performed with PE/FITC-conjugated mAbs for 30 min at 4°C. Controls were stained with labeled isotype-matched control mAbs (IgG1 PE, BD Biosciences; IgG2a FITC, DakoCytomation, Hamburg, Germany). Flow cytometric analysis was performed using the FACS Calibur apparatus and CellQuest software from BD Immunocytometry Systems (San Jose, CA).

T. gondii culture and proliferation assay

T. gondii tachyzoites of the BK strain (a gift from H. M. Seitz and M. Saathoff, Institute for Medical Parasitology, University of Bonn, Bonn, Germany) were maintained in L929 mice fibroblasts in IMDM containing 5% FCS. T. gondii tachyzoites expressing yellow fluorescent protein (YFP) were maintained in culture under similar conditions. BK-Toxoplasma were transfected with a YFP plasmid modified from the plasmid, tubYFP-YFP/sagCAT, originally a gift of B. Striepen (Center for Tropical and Emerging Global Diseases, University of Georgia, Athens, GA), and described by Gubbel et al. (20). This plasmid has a Toxoplasma protein, designated by us as B4, replacing the YFP1 locus in the plasmid (a kind gift from A. Aillyati, Institute of Medical Microbiology, University of Düsseldorf, Düsseldorf, Germany). In experiments determining Toxoplasma proliferation in DC, the cells were infected with T. gondii at a multiplicity of infection of 5 tachyzoites per DC (MOI, 5). Growth of Toxoplasma was measured by the [H]thymidine incorporation method (21). Twenty-four hours after infection, the cells were pulsed with 0.012 MBq of [H]thymidine (Amersham Biosciences) and cultured for an additional 24 h. Host cells and Toxoplasma were lysed by freeze thawing, and [H]thymidine incorporation was measured using a beta counter (1205 Betaplate; PerkinElmer, Jugenheim, Germany). In experiments aimed at determining the permissiveness of the iDC or mDC, the cells were infected with YFP-Toxoplasma at an MOI of 50 for 10 min at 37°C, and thereafter the extracellular Toxoplasma was removed by washing the cells in PBS. DC were stained with PE-labeled CD83 and then subjected to FACS analysis, and the proportion of infected (YFP-positive) DC was determined.

Determination of apoptosis

Annexin FACS analysis. Apoptosis was evaluated using the annexin V-binding assay kit (FACS annexin V-FTTC apoptosis detection kit; R&D Systems), according to the manufacturer’s protocol. Briefly, 1 × 10⁵ DC were washed in PBS and incubated with 0.25 µg/ml annexin V FITC in a calcium-containing buffer. Necrotic cells were detected using 0.5 µg/ml propidium iodide. After incubation for 15 min at room temperature, cells were washed and analyzed by flow cytometry, as described above.

Caspase-3 colorimetric assay. Apoptosis was further evaluated using the caspase-3 colorimetric assay kit (R&D Systems), according to the manufacturer’s protocol. Briefly, 1 × 10⁵ cells were incubated in lysis buffer and centrifuged (10,000 × g, 4°C, 1 min). Supernatants were collected, and the enzymatic activity was determined photometrically.

Array analysis of infected and noninfected iDC and mDC

Six hours after infection with T. gondii, extracellular parasites were removed by washing in PBS. Total RNA of mDC and iDC was isolated by density ultracentrifugation (100,000 × g, 16 h), followed by guanidinium-isothiocyanate (Merck, West Point, PA) lysis of the cells. Any remaining genomic DNA was degraded by digestion with DNase I (Applied Biosystems, Foster City, CA). RT-PCR was performed with cDNA labeling kit and human cytokine-specific primers (both R&D Systems). During reverse transcription, cDNA was labeled by incorporation of [3P]-labeled dCTP.

Specific activity of labeled cDNA was determined, and equal amounts of DC cDNA were used to hybridize the cytokine cDNA expression arrays (R&D Systems). Before hybridization, arrays were prehybridized with 100 µg/ml sonicated salmon sperm DNA (R&D Systems) for 1 h at 58°C. Hybridization was conducted overnight in a water bath at 58°C. The arrays were washed twice with 0.1× standard saline citrate/EDTA, 1% SDS, and 0.1× standard saline citrate phosphate/EDTA, 1% SDS. The cDNA arrays were exposed to a phosphor imager screen (BAS; Fuji Photo Film, Düsseldorf, Germany) for 24 h. Transcription of candidate genes was checked by RT-PCR using specific primer pairs.

RT-PCR analysis

Total RNA of infected and noninfected cells was prepared, as described above. The RT-PCR was performed using the Advantage RT-PCR Kit (BD Clontech, Palo Alto, CA). One microgram of total RNA was reverse transcribed in a 20 µl final volume containing 1 µl oligo(dT) primer. The reagents for PCR were as follows: 20 nmol each primer; 10 mM each of dGTP, dCTP, dATP, and dTTP; 1.5 mM MgCl₂ and 1 U of Taq polymerase (all PerkinElmer, Wellesley, MA); and distilled water to a volume of 50 µl. The PCR reagents were subjected to 35 cycles of: denaturing (45 s, 95°C), annealing (30 s), and extension (2 min, 72°C). TNF-α (product size, 414 bp), and GAPDH (product size, 947 bp) primers were from BD Clontech; TNF-R1 (product size, 447 bp) and TNF-R2 (product size, 665 bp) primers were from R&D Systems.

TNF-α ELISA

TNF-α protein was measured in the supernatants of infected and noninfected DC and monocytes using an ELISA from R&D Systems, according to the manufacturer’s instructions.

Blocking of TNF-R2 with neutralizing mAbs

Thirteen-day-old DC (1 × 10⁵ per well) were incubated in a 48-well culture plate (Costar Corning, New York, NY) with neutralizing anti-human TNF-R2 mAbs (R&D Systems) at different concentrations from 0.5 to a maximum of 7 µg for 1 h at 37°C. Subsequently, cells were infected with T. gondii in a ratio of 1:5 (MOI, 5) for an additional 24 h. Proliferation of Toxoplasma was determined by [H]thymidine incorporation, as described above.

Results

iDC inhibit the growth of Toxoplasma

Toxoplasma are generally able to proliferate in human nucleated cells with the single exception of fresh human monocytes that have been shown to exhibit a spontaneous toxoplasmostasis. Many other cells such as macrophages are only able to inhibit the growth of Toxoplasma after stimulation with IFN-γ. DC in culture were also permissive of Toxoplasma growth; however, after approximately the eleventh day in culture, this permissiveness changed and Toxoplasma proliferation was markedly reduced. Fig. 1A shows the intracellular proliferation of Toxoplasma in DC on days 7, 9, 13,
and 15 of culture. At each of these stages, the maturation status of the DC was checked by FACS, and the DC were found to have properties of iDC. Fig. 2 shows the phenotype of the iDC and mDC; the surface markers used in this study to define iDC were low HLA-DR, low CD86, low CD80, low CD40, and negative CD83. The figure shows a representative FACS of the iDC from days 7 through 15. No differences in the surface marker expression of the DC were detected in the DC on days 7, 9, 13, or 15, and in particular the DC did not mature in this time (see below, Fig. 3). We were interested to observe whether DC that were submitted to a forced maturation in vitro, by the addition of a cytokine mixture of TNF-α, IL-1, IL-6, TNF-α, and PGE2 for an additional 3 days, as described in Materials and Methods, were also able to restrict the growth of Toxoplasma. Fig. 1B shows the Toxoplasma proliferation in macrophages, iDC, and mDC (all on day 13 of culture). As observed before, there is a marked restriction of Toxoplasma growth in iDC compared with mDC and macrophages (all from the same donor).

**FIGURE 1.** iDC inhibit Toxoplasma growth. A, $1 \times 10^6$ DC on days 7, 9, 13, and 15 of culture, and B, $1 \times 10^6$ 13-day-old iDC, macrophages, and mDC (day 13) were infected with Toxoplasma at an MOI of 5, incubated for 24 h, and then pulsed with 0.012 MBq of $[^3H]$thymidine for the final 24 h of culture. Toxoplasma proliferation was determined by beta counting. Representative data (±SD of triplicate determination) of three similar experiments are shown.

**FIGURE 2.** FACS analysis of iDC and mDC. Monocyte-derived DC were split at day 10 of culture, and one-half treated additionally with IL-1, IL-6, TNF-α, and PGE2, for an additional 3 days (thin line). (The bold line represents the iDC.) Thereafter, the iDC and mDC were stained with FITC (HLA-DR and CD86) or PE (CD83, CD40, CD11b)-conjugated mAbs and analyzed using a FACS Calibur apparatus. The cells were gated according to forward and side scatter of unstained cells. The broken line represents the isotype-matched Abs to irrelevant Ag. The results show a typical analysis of at least 10 different preparations.

iDC and mDC are equally permissive to infection by Toxoplasma

iDC are especially efficient at phagocytosis, an attribute that is lost in the maturation process. In addition, Toxoplasma are able to invade nucleated cells in an active process that ensures entry into the cell within a few minutes. It is therefore possible that differences in the susceptibility or permissiveness of the DC result in the observed difference in Toxoplasma growth in the iDC and mDC, dependent on the mode of entry into the cell. To demonstrate the presence of such a difference in susceptibility to Toxoplasma infection, we infected DC with YFP-expressing Toxoplasma and analyzed the cells in FACS after staining with PE-labeled CD83. In both iDC and mDC, the percentage of infected cells was determined by calculating the cells staining for YFP and for the CD83 molecule. Cells thus negative for CD83 and positive for YFP represented infected iDC and cells double positive, infected mDC. In Fig. 4, the mean of paired results of the percentage of infected iDC and mDC is shown (iDC, 46.6 ± 17.8%; mDC, 40.0 ± 13.5%). Thus, we were able to show that the resistance to Toxoplasma growth in iDC is not due to the iDC being less permissive to infection than the mDC. This experiment is especially suited to demonstrate any differences in infection in that the iDC and mDC can be infected with Toxoplasma in a single well, thereby creating identical experimental conditions for both cell types. These results show that the resistance of iDC to Toxoplasma growth is not secondary to reduced cell susceptibility to infection.

**Both iDC and mDC demonstrate an IFN-γ-induced toxoplasmostasis**

To determine whether the DC were capable of inhibiting the growth of Toxoplasma after stimulation with IFN-γ (as are almost all effector cells), we incubated the DC with different concentrations of IFN-γ. After 24 h, the DC were infected with Toxoplasma
(MOI, 5), and Toxoplasma growth was measured, as described. Fig. 3 shows the results of a typical experiment in which the growth of the Toxoplasma is displayed relative to that in iDC. The parasite proliferation in iDC is approximately one-half that of the mDC, and both the iDC and mDC show an IFN-γ-induced toxoplasmostasis. It is interesting to note that the toxoplasmostasis of the mDC does not reach levels seen in iDC (or, for that matter, of macrophages). These results also show that the iDC possess both an IFN-γ-independent as well as an IFN-γ-dependent toxoplasmostatic effector mechanism. To attempt to determine which mechanism may be involved, we incubated the iDC with and without IFN-γ in the presence or absence of different oxygen radical scavengers, such as mannitol, 1,4 diazabicyclo [2,2,2] octane, superoxide dismutase, and benzoic acid, and were unable to show any effect on either the spontaneous toxoplasmostasis of iDC or the IFN-γ-induced toxoplasmostasis of iDC or mDC. Furthermore, we were unable to detect a production of NO or H₂O₂ using FACS analysis of dichlorofluorescein-treated iDC and mDC (data not shown). This experiment shows that both the iDC and mDC exhibit an IFN-γ-induced Toxoplasma growth-inhibitory effect. The growth permissiveness of the mDC relative to iDC is preserved even after stimulation with IFN-γ, and the iDC exhibit an IFN-γ-dependent and an IFN-γ-independent toxoplasmostasis.

iDC remain immature after infection with T. gondii
iDC are well suited to phagocytosis of pathogens, after which they migrate to the regional lymph nodes from the periphery and concomitantly develop an mDC phenotype more suited to Ag presentation. Many different Ags and pathogens have been shown to influence DC maturation such as LPS, HIV, Mycobacterium tuberculosis, and Gram-negative bacteria such as Escherichia coli. This has not been shown for Toxoplasma, and it was of interest to determine whether Toxoplasma-infected iDC undergo maturation and whether this Toxoplasma-driven process may be in part responsible for the growth inhibition of the parasite. iDC infected with Toxoplasma for 24 h were analyzed for markers of maturation, and the results are shown in Fig. 5. As can be seen, infection of iDC with Toxoplasma does not induce maturation of the cells.
Toxoplasma growth inhibition is not due to apoptosis of the infected iDC

Apoptosis of an infected cell and subsequent ingestion by other APC is an effective immune mechanism toward eliminating the pathogen and stimulation of the adaptive immune system. If *Toxoplasma*-infected iDC undergo apoptosis, parasite growth may be inhibited, and thus a difference in the rate of apoptosis between iDC and mDC could account for the toxoplasmostasis observed in iDC. Apoptosis was measured by determining both the caspase-3 activity and the binding of annexin V to the cell surface of infected and noninfected DC. In both experiments, the iDC and mDC were infected for 24 h, and thereafter, apoptosis was determined. Non-infected cells were treated in the same way as the infected cells, and as a control apoptosis was induced in U937 cells with TNF-α plus cycloheximide.

In Fig. 6A, the binding of annexin shows that both a so-called early and late apoptosis in the iDC was not increased after infection with *Toxoplasma*. In both iDC and mDC, the late apoptosis was significantly greater; however, infection with *Toxoplasma* did not result in an increased apoptosis. Interestingly, in the mDC, there was a marked increase in the early apoptosis induced by *Toxoplasma* infection; however, this was not enough to offset the total *Toxoplasma* proliferation that presumably occurred in the remaining nonapoptotic cells. These results were confirmed by the caspase-3 activity assay (Fig. 6B), in which a similarly greater apoptosis was observed in mDC after infection. These results suggest that the observed *Toxoplasma* growth restriction is not secondary to a selective depletion of the host cell in iDC cultures due to apoptosis induced by *Toxoplasma* infection.

**TNF-R2 gene expression in DC is dependent on maturity and Toxoplasma infection**

mDC and iDC were incubated for 6 h with *Toxoplasma* tachyzoites (MOI, 5); thereafter, the extracellular *Toxoplasma* was removed by washing the cells, and the total RNA was extracted. Cytokine arrays were hybridized with cDNA from the infected and uninfected iDC and mDC. A comparison of the expression patterns identified the differential expression of the TNF-R2 gene. RT-PCR analysis confirmed this result, as seen in Fig. 7A. A signal for TNF-R2 in iDC is present only after infection, and furthermore, mDC express TNF-R2 neither in the infected nor the uninfected state. In contrast, TNF-R1 is expressed in both iDC and mDC as well as in monocytes, and moreover, the state of infection has no effect on the expression of this gene.

To ensure that the expression of TNF-R2 in mDC did not occur at an earlier time point and therefore was not detected, we harvested RNA from DC at 30 min, 1 h, 2 h, 4 h, and 6 h postinfection, and performed RT-PCR to detect TNF-R2 mRNA. As shown in Fig. 7B, there is no detectable mRNA for TNF-R2 in mDC, whereas expression in iDC is detectable at 1 h postinfection, reaching a maximum at 4 h. The results demonstrate that TNF-R2 expression in iDC is a result of infection with *Toxoplasma*, and that the mDC do not express TNF-R2, whereas TNF-R1 is equally expressed in all cells tested. Furthermore, TNF-R2 is also expressed in monocytes after infection with *Toxoplasma*, a finding that is interesting in view of the fact that monocytes also exhibit an innate toxoplasmostatic effect.

**TNF-α expression by iDC, mDC, and monocytes is independent of Toxoplasma infection**

TNF-α has been shown in mice to be important in the control of *Toxoplasma* infection, although its role in human toxoplasmosis is perhaps secondary to IFN-γ. To determine whether the iDC were secreting TNF-α after *Toxoplasma* infection, we measured TNF-α expression and also protein in the culture supernatants before and after infection. Fig. 8A shows the TNF-α mRNA in infected and noninfected iDC, mDC, and monocytes. No obvious difference in the expression of TNF-α is seen before and after infection and between the different cell types. We detected very small amounts of TNF-α protein in the supernatants of all cells (50–400 pg/ml), although this did increase upon infection of iDC and mDC (Fig. 8B). This is very low in comparison with TNF-α levels normally detected in LPS-stimulated cells (usually at ng/ml levels). Furthermore, we incubated iDC with neutralizing anti-TNF-α Abs for 30
min before infection with Toxoplasma and were unable to demonstrate any effect on the toxoplasmostasis in the iDC (data not shown). The expression of TNF-α and the detection of low amounts of TNF-α protein in supernatant of all cells, both before and after infection, do not support a major role of TNF-α in the innate toxoplasmostasis of iDC, and the additional failure of neutralizing TNF-α Abs to abrogate this effect at the very least does not increase the likelihood of a possible role for TNF-α.

Toxoplasmostasis is inhibited by neutralizing the TNF-R2

TNF-α has been shown to play a supportive role in the defense against Toxoplasma; however, there was no effect in the absence of IFN-γ. Because we were unable to detect IFN-γ in the supernatants of iDC and equally could not detect mRNA for IFN-γ, we assume that IFN-γ does not play a part in the described toxoplasmostasis of iDC (data not shown). It was possible that the expression of TNF-R2 was merely marker or signal for an immunological process taking place in the infected cell and in itself had no, or little, direct role. We thus used neutralizing anti-TNF-R2 mAbs to determine whether TNF-R2 had a functional role in the toxoplasmostasis in iDC. Fig. 9A shows the effect of incubating the iDC with anti-TNF-R2 Abs at varying concentrations before infecting the cells with Toxoplasma. The Toxoplasma growth is depicted relative to that of untreated iDC (given the value 1). Increasing concentrations of neutralizing Ab result in a decreased toxoplasmostatic effect. At 7 μg/ml, the toxoplasmostasis is almost completely abrogated. In contrast, the Abs have no effect on mDC, as is shown in Fig. 9B. To exclude an unspecific Ab effect, we incubated the iDC and mDC with Abs of the same Ig class as that of the anti-TNF-R2 Ab, which recognize a nonhuman epitope. We observed no effect (data not shown). These results demonstrate a functional role for TNF-R2 by showing an abrogation of the toxoplasmostasis by use of neutralizing TNF-R2 Abs in a concentration-dependent manner.
the growth of more, we show that maturation of the iDC that are able to restrict cell occurred fairly constantly at about day 11 of culture. Further-
study the in
fluence of DC maturation on their response to
are not yet clear, we felt it would be interesting and appropriate to
and many other intracellular pathogens are controlled
Toxoplasma
Considering that DC are among the
B
FIGURE 9. TNF-R2 has a functional role in toxoplasmostasis. DC (A, iDC; B, mDC) were incubated for 1 h with neutralizing anti-TNF-R2 mAb, and thereafter infected with Toxoplasma (MOI, 5), incubated for an additional 24 h, and then pulsed with 0.012 of MBq [³H]uracil for the final 24 h of culture. Toxoplasma proliferation was determined by beta counting. Data are shown relative to the Toxoplasma growth in untreated iDC, which is assigned a value of 1. Representative data (±SD of triplicate determinations) of at least three similar experiments are shown.

Discussion
Considering that DC are among the first cells to come into contact with Toxoplasma, that the cellular immune response is essential to the mounting of this response, and that the mechanisms by which Toxoplasma and many other intracellular pathogens are controlled are not yet clear, we felt it would be interesting and appropriate to study the influence of DC maturation on their response to Toxoplasma. We observed early on that the Toxoplasma proliferation in DC was reduced after a certain time of culture. The conversion from a Toxoplasma growth-permitting cell to a growth-restricting cell occurred fairly constantly at about day 11 of culture. Furthermore, we show that maturation of the iDC that are able to restrict the growth of Toxoplasma using a mixture of TNF-α, IL 6, IL-1, and PGE₂, abrogated this toxoplasmostatic effect of the DC. In both mouse and human models of Toxoplasma infection, the major stimulus causing a toxoplasmostasis is, regardless of the mechanism induced, IFN-γ. It has been clearly demonstrated in IFN-γ knockout mice that deficiency of IFN-γ results in a lethal infection (22, 23) and, in humans, IFN-γ stimulates many cells such as macrophages (9), endothelial cells (24), and astrocytes (25) to exhibit a toxoplasmostatic effect. To date, a spontaneous growth restriction has only been described in fresh human monocytes (14, 26). Wilson and Remington (14) described the Toxoplasma growth-inhibitory effect of monocytes infected within the first hour of culture. This activity was later further characterized to be present only in the adherent population of monocytes and not to be present in nonadherent monocytes (27). This toxoplasmostatic activity was shown to be IFN-γ-independent and partly due to reactive oxygen species such as OH⁻, O₂⁻, and H₂O₂ (10). There was, however, some activity against Toxoplasma that was independent of reactive oxygen species. In the present study, we describe for the first time a spontaneous inhibition of Toxoplasma growth in iDC. We show that the toxoplasmostasis is not due to the presence of IFN-γ in the cultures because we were neither able to detect IFN-γ in the culture supernatants nor the presence of contaminating IFN-γ-producing T cells or NK cells (data not shown). In addition, we show that the growth inhibition is not due to increased apoptosis of the iDC. We also show that the infected cells do not, after infection, develop a mature phenotype, as measured by CD83, CD40, CD86, and HLA-DR up-regulation. This is in contrast to Subauste and Wessendarp (28), who demonstrated that the costimulatory molecule markers CD86, CD40, and CD80 were up-regulated after infection with Toxoplasma, but not the maturation marker CD83. This apparent discrepancy may be due to the fact that our iDC are held for longer in culture than the 7-day-old DC used in the study by Subauste and Wessendarp (28). This explanation would be supported by the fact that the cell age appears to be critical for the toxoplasmostatic activity, which suggests a selection of a subpopulation of iDC or a further differentiation into a subtype as yet uncharacterized.

Our preliminary investigations in determining an underlying mechanism for the toxoplasmostatic activity of the iDC showed us that the known effector mechanisms described in cells of the innate immune system were not responsible. Using specific inhibitors, we excluded reactive effector mechanisms, NO, and IDO activity as a source of the growth control of the intracellular Toxoplasma. We therefore opted for another approach to search for the mechanism and used cytokine gene expression arrays using cDNA from iDC, mDC, and monocytes both before and after infection with Toxoplasma. Investigation of the differential cytokine gene expression led to the observation that after infection both iDC and monocytes up-regulate their expression of TNF-R2 (p75), which is not detectable in sensitive RT-PCR in the uninfected cells, and in addition, neither detectable in infected nor uninfected mDC or macrophages. This was in contrast to the ubiquitous expression of TNF-R1 in all four cell types tested in both infected and uninfected states. This study showed a maximal expression of TNF-R2 after infection at 4 h in both iDC and monocytes, which correlates well with the results from Belloni et al. (18), who demonstrated a TNF-R2 up-regulation in Toxoplasma-infected THP-1 cells, also 3–4 h after infection with Toxoplasma. This is in contrast to another report that showed no increased expression of TNF-R2 after infection with Toxoplasma (29). Both of these studies used THP-1 cells in contrast to the iDC used in this study. The role of TNF-α receptors in Toxoplasma has been best investigated in the mouse model, especially using TNF-R1 and TNF-R2 knockout mice. TNF-R1 knockouts were susceptible to chronic toxoplasmosis, whereas they were resistant to the acute infection (17, 16). The
TNF-R2 knockout mice were not more susceptible to acute or chronic Toxoplasma infection than the wild-type mice, thus putting into question the importance of TNF-R2 for the control of toxoplasmosis in mice. The expression of TNF-R2 on mouse endothelial cells was shown to play a major causative role in the pathology of cerebral malaria caused by infection of mice with Plasmodium berghei Anka (30, 31). In this study, the TNF-R2-deficient mice were not susceptible to cerebral malaria, and the authors postulated a role of TNF-R2 in the metabolic homeostasis of the brain endothelial cells, although a specific role of TNF-R2 was not determined. A number of other studies have shown a role for TNF-R1 in the control of other intracellular pathogens such as Listeria monocytogenes (32), Leishmania major (33), and Mycobacterium tuberculosis (34). In contrast to this, the function of TNF-R2 in infection has not been entirely elucidated. The affinity for the two known ligands, TNF-α and lymphotoxin α, is far higher for TNF-R2 than TNF-R1, although the signaling occurs for the most part through TNF-R1. The TNF-R2 may have a central role in the passing on of the ligand to the TNF-R1, as it also has a higher dissociation constant than does TNF-R1 (19). Studies in human cells are, however, lacking. To our knowledge, this is the first description of a possible role for TNF-R2 in the control of parasite infection in humans. Although it is not clear exactly what function TNF-R2 has, the expression alone is obviously not merely a signal event for another mechanism ultimately responsible for the control of parasite intracellular growth. This is clearly shown by the abrogation of the fecundity of Toxoplasma in iDC when the cells are treated with neutralizing anti-TNF-R2 Ab. The obvious question is then what role does TNF-α have in the system? Our data show that the production of TNF-α by both iDC and mDC is very low, although it does increase in both cell types after infection. We were unable to show that neutralizing TNF-α using Abs had any effect in the control of growth in iDC. It does remain possible that membrane-bound TNF-α (mTNFα) or autocrine intracellular TNF-α has a key role, and that we are unable to neutralize this with extracellular Abs. The iDC in culture do tend to clump together in large aggregates after infection, which implies a cell-cell contact suggesting mTNFα as a possible initiator or regulator of Toxoplasma growth control. What effect TNF-α has, either in terms of autocrine secretion and stimulation or in the membrane bound, remains open and requires further study. It is interesting to note, however, that a role for TNF-α alone in the control of Toxoplasma has never been shown in humans, despite the clear synergistic effect it has with IFN-γ in this respect. We were unable to detect any IFN-γ in the cultures; however, we demonstrate that both the iDC and mDC are IFN-γ responsive and can inhibit the growth of Toxoplasma after stimulation.

In conclusion, our data show for the first time a spontaneous toxoplasmastosis in human iDC of a certain age, which is matched by the effect in fresh monocytes, as has been described in the literature. We show that TNF-R2 is expressed in the cells exhibiting the Toxoplasma growth control and not in those cell types tested that do not exhibit toxoplasmastosis, and furthermore, that blocking of the TNF-R2 leads to the abrogation of the parasite growth control. Further investigations are underway in our laboratory to attempt to elucidate the underlying mechanism behind the expression of the TNF-R2 and the role of soluble TNF-α and/or mTNFα, and also to try and determine which subpopulation, if any, of DC is involved.

References

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