Major role for CD8\(^+\) T cells in the protection against *Toxoplasma gondii* following dendritic cell vaccination

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**SUMMARY**

*Toxoplasma gondii* is the causative agent of toxoplasmosis, a worldwide zoonosis for which an effective vaccine is needed. Vaccination with pulsed dendritic cells is very efficient but their use in a vaccination protocol is unconceivable. Nevertheless, unravelling the induced effector mechanisms is crucial to design new vaccine strategies. We vaccinated CBA/\(\bar{J}\) mice with parasite extract-pulsed dendritic cells, challenged them with *T. gondii* cysts and carried out in vivo depletion of CD4\(^+\) or CD8\(^+\) T lymphocytes to study the subsequent cellular immune response and protective mechanisms. CD4\(^+\) lymphocytes were poorly implicated either in spleen and mesenteric lymph node (MLN) cytokine secretion or in mice protection. By contrast, the increasing number of intracerebral cysts and depletion of CD8\(^+\) cells were strongly correlated, revealing a prominent role for CD8\(^+\) lymphocytes in the protection of mice. Splenic CD8\(^+\) lymphocytes induce a strong Th1 response controlled by a Th2 response whereas CD8\(^+\) cells from MLNs inhibit both Th1 and Th2 responses. CD8\(^+\) cells are the main effectors following dendritic cell vaccination and *Toxoplasma* infection while CD4\(^+\) T cells only play a minor role. This contrasts with *T. gondii* infection which elicits the generation of CD4\(^+\) and CD8\(^+\) T cells that provide protective immunity.

**Keywords** CD8\(^+\) T cells, dendritic cell, toxoplasma gondii, Vaccination

**INTRODUCTION**

*Toxoplasma gondii* is an obligate intracellular protozoan that infects one-third of the world population. Asymptomatic in immunocompetent hosts, toxoplasmosis has severe consequences in immunosuppressed individuals and can even lead to death (1,2). Congenital toxoplasmosis causes development of sequelae later in life, including chorioretinitis, hearing loss or mental retardation (3). Recent findings strongly suggest a link between *T. gondii* seropositivity and schizophrenia (4). In animals, *T. gondii* is responsible for numerous abortions thus causing substantial reproductive and economic losses (5). Additionally, these infected animals are a parasitic reservoir involved in human contamination.

Primary infection with *T. gondii* results in the setting of both humoral and cell-mediated immune responses and confers long-term protection. This suggests that the development of an efficient vaccine is a realistic goal. Therefore, many studies have investigated possible solutions for an efficient vaccine (6,7). The elicited immune response following a *T. gondii* infection first needs to be clearly defined before a vaccine can be developed.

Host resistance seems to occur via synthesis of IFN-\(\gamma\) by NK cells and adaptive T lymphocytes (8). Following the infection, macrophages synthesize TNF-\(\alpha\) and IL-12 which induce NK cells to secrete IFN-\(\gamma\). The combined action of IL-12 and IFN-\(\gamma\) induce a strong differentiation of T-helper precursors into Th1 lymphocytes. These CD4\(^+\) T cells then synthesize large amounts of IFN-\(\gamma\) and IL-2. These two cytokines finally induce CD8\(^+\) T lymphocytes proliferation and IFN-\(\gamma\) secretion (8). Thus protection against *T. gondii* infection is mainly attributed to cell-mediated immunity. Previous studies have shown that both CD4\(^+\) and CD8\(^+\) T-cell subtypes are involved in the protection. The relative contribution of these two populations was investigated by adoptive transfer or *in vivo* depletion (9). All these data suggest a prominent role of CD8\(^+\) cells with a supporting role for CD4\(^+\) cells.
during the acute phase as well as during the chronic phase of infection. If IFN-\(\gamma\) is the major cytokine of resistance to \(T. gondii\) (10), IL-12 is a crucial initiation cytokine to trigger an efficient cell-mediated immunity. Indeed, IL-12 is a major cytokine secreted by neutrophils, macrophages and dendritic cells (DCs), that triggers the early IFN-\(\gamma\) secretion following \(T. gondii\) infection. DCs are the first producers of IL-12 in response to \(T. gondii\) antigens (11). We have previously demonstrated that primary DCs or a DC line (SRDCs) pulsed \textit{ex vivo} with \(T. gondii\) antigen induced a protective cellular immune response to a virulent oral challenge (12,13). Although DC vaccination is very efficient, its use in a vaccination protocol is unconceivable. However, the description of the effector mechanisms induced following such a vaccination is a key step in designing new vaccines that target \textit{in vivo} endogenous DC populations. This targeting would aim at triggering the previously described efficient immune response.

In this study, for the first time, we investigated the role of two main lymphocytic populations, CD4\(^+\) and CD8\(^+\), in the protective response to \(T. gondii\) in CBA/J mice, chronically infected, following a DC vaccination. We demonstrated that CD4\(^+\) T lymphocytes were not implicated either in spleen or mesenteric lymph node (MLN) cytokine secretion or in long-term protection of mice. On the contrary, CD8\(^+\) T lymphocytes appeared as the main effectors, inducing a strong Th1 response in spleen while inhibiting both Th1 and Th2 responses in MLNs.

**MATERIALS AND METHODS**

**Materials**

All reagents for cell culture were obtained from Invitrogen (Carlsbad, CA, USA) and Sigma-Aldrich (St-Louis, MO, USA); enzyme-linked immunosorbent assay (ELISA) reagents and recombinant cytokines were from BD Pharmingen (San Diego, CA, USA), unless otherwise stated.

**Mice**

Seven-week-old CBA/J mice (H-2k) (Janvier, Le Genest Saint Isle, France) were maintained in pathogen-free conditions. Groups of 12 mice were used for the chronic toxoplasmosis model.

**Parasites and \(T. gondii\) extract**

Type I strain RH tachyzoites are highly virulent for mice and infection with a single parasite results in the death of the animal. RH tachyzoites were used to prepare \(T. gondii\) antigen because they multiply easily in cell culture. They were harvested from infected monolayers of human foreskin fibroblasts Hs 27 (ATCC CRL-1634) and were the source of \(T. gondii\) extract (TE) (12).

Type II strain 76K are relatively avirulent for mice and are predominant in human and sheep congenital toxoplasmosis. Cysts of the \(T. gondii\) strain 76K were used for oral challenge because infection results in establishing a chronic infection by cyst formation. They were obtained from brains of CBA/J mice orally infected by gavage 1 month earlier with 80 cysts.

**Immunizations**

SRDC (H-2k) (13), were cultured in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 5% heat-inactivated foetal calf serum (FCS), penicillin (50 U/mL)/streptomycin (50 \(\mu\)g/mL), \(\beta\)-mercaptoethanol (50 \(\mu\)M) and \(\alpha\)-glutamine (2 \(\mu\)M). Cells were pulsed with 50 \(\mu\)g/mL of TE (TE-SRDC) or unpulsed (NP-SRDC) and were harvested after 24 h and resuspended (2.5 \(\times\) \(10^6\) cells/mL) in Roswell Park Memorial Institute (RPMI). Mice were subcutaneously injected with 5 \(\times\) \(10^5\) TE-SRDC or NP-SRDC. A second injection was carried out 15 days later. Control mice were untreated.

**Efficiency of immunizations**

Two weeks after the second injection, efficiency of immunizations was tested. Blood samples were collected from the retro-orbital sinus of mice and spleens were harvested as described in section ‘Measurement of cytokine and nitric oxide concentrations’. Immunization efficiency was tested by ELISA on \(T. gondii\)-specific IgG and by specific proliferation and cytokine secretion assays on spleen cells. 96-well plates were coated with 10 \(\mu\)g/mL TE and incubated overnight at 4\(^\circ\)C. Wells were then saturated with 4% bovine serum albumin and processed as previously described (14). Sera were diluted in 1 : 50, 1 : 100 and 1 : 200.

Spleen cells were collected and prepared as described in section ‘Measurement of cytokine and nitric oxide concentrations’. Cell culture supernatants were tested for the presence of IFN-\(\gamma\), IL-2, IL-10 and IL-4 (see Measurement of cytokine and nitric oxide concentrations). The measurement of antigen-specific proliferative response was also assessed on these cells (12).

**Depletion and Toxoplasma challenge**

To obtain monoclonal antibodies (mAbs) directed against CD4\(^+\) and CD8\(^+\) cells, GK1-5 (rat IgG\(_{2b}\), ATCC TIB-207) and H35-17.2 (rat IgG\(_{2b}\)) (15), hybridomas were cultured.
in RPMI/DMEM (1:1) supplemented with 20% or 10% FCS, respectively. Supernatants were collected, centrifuged at 1500 g for 20 min, filtered using a membrane with 0.45 μm pores to remove residual cell fragments, and were purified using HiTrap Protein G affinity columns (GE Healthcare, Uppsala, Sweden).

Two weeks after the second immunization, mice were intraperitoneally injected on three consecutive days with 0.6 or 0.3 mg of anti-CD4 or anti-CD8 mAb, respectively. Mice were then injected every six days until they were killed.

On the third day of depletion, mice were challenged by gavage with 80 cysts from the 76K strain of *T. gondii*. Depletion was assessed by reacting spleen and MLN cells of mice, killed at different times, with a fluorescein isothiocyanate-conjugated mAb against CD4 (GK1.5) or CD8 (53-5-8) (all from BD PharMingen). Before all labelling experiments, Fc receptors were blocked by incubating cells with the 2-4G2 mAb for 10 min. Unrelated isotype-matched mAbs were used for a control. Analysis was performed using FACSscan apparatus (Becton Dickinson & Co., San Diego, CA, USA) and CellQuest v3.3 software (Becton Dickinson). The efficacy of depletion of MLN and spleen cells was ≥87% and 90% for CD4⁻ and ≥96% and 99% for CD8⁻ cells, respectively.

Measurement of cytokine and nitric oxide concentrations

Spleen cells and MLN cells were collected at the end of the experiment and pressed through a stainless steel mesh. Single-cell suspensions were obtained by filtration through nylon mesh to remove tissue debris. The spleen erythrocytes were destroyed by hypotonic shock, and spleen and MLN cells were resuspended in RPMI 1640 supplemented with 5% FCS, HEPES (25 mM), L-glutamine (2 mM), sodium pyruvate (1 mM), β-mercaptoethanol (5 × 10⁻⁵ M) and penicillin (50 U/mL)/streptomycin (50 μg/mL). Cells were then cultured in 24-well plates at 10⁶ cells per well in 1 mL of culture medium, alone or containing TE (10 μg/mL). Plates were incubated for 4 days in 5% CO₂ at 37°C. Cell supernatants were harvested between 24 h and 4 days and IFN-γ, IL-2, IL-10, IL-4 and nitric oxide (NO) concentrations were measured.

Cytokine concentrations were evaluated using commercial ELISA kits according to the manufacturer’s instructions. They were determined from standard curves constructed with known amounts of mouse recombinant IL-2, IL-4, IL-10 and IFN-γ. The sensitivity limits for the assays were 3-1 pg/mL for IL-2, 7-8 pg/mL for IL-4 and 31-3 pg/mL for IL-10 and IFN-γ.

Nitric oxide concentration was evaluated using the Griess test. Briefly, Griess reagents (sulfanilamide 1% and naphthylethlenediamine 0.1%, ratio 1:1) (Sigma) were added to the culture supernatants. Absorbance was measured at 540 nm. NO concentration was determined using a standard curve established with a 20 nM sodium nitrite solution. The sensitivity limit was 2 μM.

Results are presented as mean ± SEM.

Intracellular IFN-γ assay

The frequency of IFN-γ producing CD8⁺ and CD4⁺ splenocytes of immunized and challenged CBA/J mice (three per group) was detected 1 month after oral challenge. CD8⁺ or CD4⁺ cells were isolated and purified by positive selection using microbeads coated with mAbs to CD8 or CD4 according to the instructions provided by the manufacturer (Miltenyi Biotec, Auburn, CA, USA) to yield populations consisting of >98% CD8⁺ or CD4⁺. CD8⁺ or CD4⁺ T cells were seeded at 10⁶ cells per well in a 24-well plate and were stimulated with 1⁰ irradiated unpulsed or TE-pulsed SRDC cells (5000 Rads) for 1 h at 37°C followed by an additional 4 h stimulation in the presence of 10 μg/mL brefeldin A (Golgi Plug: 2301 KZ, Pharmingen) per 2 × 10⁶ cells. Permeabilization of the cells was performed using a proprietary solution (Becton Dickinson). Cells were stained for surface CD8 or CD4 and intracellular IFN-γ expression using directly conjugated mAbs according to the manufacturer’s recommendations, and isotype-matched negative controls were used for all antibodies. For analysis, 150 000–200 000 cells were acquired on a FACSCalibur flow cytometer (BD Biosciences, Le Pont de Claix, France).

Effecter function of splenic CD8⁺ T cells

One of the aims of this study was to determine whether CD8⁺ T cells stimulated by pulsed dendritic cells display effector functions. So we have studied the capacity of splenic CD8⁺ T cells to produce IFN-γ and IL-2 and their cytolytic activity. Two weeks after the second immunization, CD8⁺ T cells from spleen were purified by positive selection, using anti-mouse CD8 (Ly2) paramagnetic beads (Dynal Biotech, Invitrogen, Cergy Pontoise, France), yielding populations consisting of >98% CD8⁺ cells. Cells were suspended in RPMI 1640 (GIBCO, Invitrogen, Cergy Pontoise, France) supplemented with 5% FCS, 25 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 5 × 10⁻⁵ M β-mercaptoethanol, and 100 μg/mL penicillin/streptomycin.

For cytotoxicity assay SRDC cells used as target cells were maintained at 37°C in culture medium and were detached by trypsin treatment at confluence. The cells were washed three times in Hank’s buffered salt solution (HBSS), resuspended in culture medium and dispensed at a density of 3 × 10⁶ cells per well in round-bottomed tissue culture plate (Falcon, Lincoln Park, NJ, USA), with
or without TE. They were incubated for at least 4 h and were then radio-labeled with $^{51}$Cr (1 μCi/well; specific activity 469 mCi/mg; Dupont NEN, MA, USA) for 3 h. The cells were washed until the culture supernatant contained $<$500 cpm radioactivity and the radio-labeled target cells were then incubated with CD8$^+$ T cells at various E:T ratios, in 200 μL of culture medium. Culture plates were centrifuged at 200 g for 2 min, and were then incubated for 4 h. At the end of the assay, the plates were centrifuged at 200 g, and 100 μL of supernatant was harvested from each well and the radioactivity released was determined by scintillation counting (Hewlett-Packard, Les Ulis, France). The percentage of specific $^{51}$Cr release was calculated as: [mean cpm of sample tested – mean cpm of spontaneous release] x 100/[mean cpm of maximal release – mean cpm of spontaneous release] x 100.

For cytokine assay CD8$^+$ T were cultured in 24-well plates (10$^6$ cells/well) alone or with 10 μg/mL TE or with SRDC alone or with 10 μg/mL TE-pulsed SRDC (2·10$^5$ cells/well). Culture supernatants were harvested at 24 and 96 h for IL-2 and IFN-γ production, respectively. IL-2 and IFN-γ concentrations were evaluated with an ELISA kit, according to the instructions of the manufacturer (BD Pharmingen).

**Evaluation of protection in mice**

Brains were harvested 1 month after oral challenge and homogenized in 5 mL of RPMI 1640 with a pestle and mortar. Cysts in brain homogenates were counted microscopically (10 $\times$ 10 μL). Results are expressed as mean ± SEM for each group.

**Statistical analysis**

Statistical analysis was performed using the Mann–Whitney U-test (InStat 2.01 software) to analyse the observed differences in cytokine productions and cyst counts. $P < 0.05$ was considered to be statistically significant.

**RESULTS**

**Efficiency of immunizations**

Two weeks after the second immunization, efficiency of immunizations was assessed at humoral and cellular levels. *T. gondii*-specific production of IgG was exclusively observed in mice immunized with pulsed DCs (data not shown).

According to our previous published results (13), we showed that the splenocytes from TE-SRDC-immunized mice were unique in their capacity to proliferate following restimulation with parasite extract and that this proliferation was dose-dependent (data not shown). The proliferative response was associated with an increased production of IFN-γ, IL-2, IL-10 and IL-4 in spleen. Theses results indicated that the cellular immune response elicited *in vivo* by TE-pulsed SRDC was correlated with a mixed Th1/Th2 response.

We concluded that immunizations were efficient and induced a specific response directed against *T. gondii*. We next tested our vaccination protocol by oral infection of mice.

**Post-infection cellular response following CD4$^+$ depletion**

Mice were killed one month after oral challenge. Splenectomys and MLNs were harvested and cells were cultured in the presence of TE (10 μg/mL) or medium alone. Supernatants were collected at various times and cytokine and NO productions were determined.

In spleens (Figure 1), IFN-γ levels were similar for CD4$^+$-depleted (NP-SRDC x-CD4) and nondepleted (NP-SRDC) splenocytes of NP-SRDC mice (14 058 and 15 082 pg/mL, respectively) and of TE-SRDC mice (25 758 and 25 380 pg/mL, respectively). Nevertheless, there is a significant difference between TE-SRDC and NP-SRDC mice (25 380 and 15 082 pg/mL, respectively; $P < 0.01$). The CD4 depletion did not impair IFN-γ secretion observed following immunization with pulsed-DCs.

There was no significant difference in IL-2 levels from depleted or nondepleted groups, either for NP-SRDC mice (15 and 23.8 pg/mL, respectively) or for TE-SRDC mice (31.1 and 28.1 pg/mL, respectively).

We could not see any statistical difference between the groups among IL-10 levels in spleens (1830 pg/mL for control mice, 1772 pg/mL for NP-SRDC mice, 1806 pg/mL for depleted NP-SRDC mice, 1944 pg/mL for TE-SRDC mice and 1825 pg/mL for depleted TE-SRDC mice).

We were unable to detect IL-4 secretion.

Nitric oxide production in spleens was similar between depleted and nondepleted groups for both NP-SRDC and TE-SRDC mice. Indeed, CD4 depletion had no effect on NO production in NP-SRDC mice [11.3 μM (depleted) and 12.3 μM (nondepleted)] as well as in TE-SRDC mice [11.5 μM (depleted) and 14 μM (nondepleted)]. Nevertheless, a significant increase was observed in TE-SRDC mice when compared to the control group (14 and 8 μM, respectively; $P < 0.05$). Thus, CD4$^+$ cells are not implicated in NO secretion in spleens.

These findings seem to indicate that CD4$^+$ lymphocytes have a minor role, during the chronic phase, in the
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Post-infection cellular response following CD8+ depletion

Splenocytes from CD8+-depleted and nondepleted NP-SRDC mice did not secrete significantly different levels of IFN-γ (18 866 and 15 082 pg/mL, respectively) (Figure 2). On the other hand, depleted TE-SRDC mice secreted significantly lower IFN-γ levels than nondepleted mice (10 254 and 25 380 pg/mL, respectively; P < 0.01). Thus, the CD8+ cell population are responsible for splenic IFN-γ synthesis during the chronic phase.

Depleted groups of both NP-SRDC and TE-SRDC mice showed a significantly lower IL-2 secretion than their nondepleted control groups (9.3 and 23.8 pg/mL in NP-SRDC mice, P < 0.05; 9.5 and 28.1 pg/mL in TE-SRDC mice, P < 0.05). CD8+ splenocytes also seem to be a major source of IL-2.

Similar changes were observed for IL-10 secretion to those observed for IL-2: depleted groups had markedly decreased IL-10 levels [100 pg/mL (depleted) and 1772 pg/mL (nondepleted) for NP-SRDC mice, P < 0.05; 1138 pg/mL (depleted) and 1944 pg/mL (nondepleted) for TE-SRDC mice, P < 0.05]. Contrary to IFN-γ, secretions of IL-2 and IL-10 do not seem to be T. gondii-specific as a decrease is observed when both TE-SRDC and NP-SRDC mice are depleted.

No IL-4 secretion was observed among the groups. These findings reveal an important role for CD8+ T splenocytes in type I cytokine secretion (IFN-γ and IL-2). The observed reduction in IL-10 following CD8 depletion secretion of type I and II cytokines following DC vaccination and challenge.

In MLNs, no statistical difference was observed between the groups whatever the considered cytokine, even concerning NO production (data not shown).

These findings suggest that CD4+ T lymphocytes are not implicated in either Th1 and Th2 cytokine secretion or nitric oxide production in MLNs.

**Figure 1** Post-infection cellular response in spleen following CD4+ depletion. CBA/J mice were immunized twice with dendritic cells pulsed (TE-SRDC) or not (NP-SRDC) with T. gondii extract (TE). Fifteen days post-immunization, mice were depleted with a monoclonal antibody (α-CD4) or not and were all orally challenged with 80 cysts from the 76K strain of T. gondii. Control mice were only infected. One month after oral challenge mice were killed. Spleen cells were cultured in the presence of parasite extract (TE). Fifteen days post-immunization, mice were depleted with a monoclonal antibody (α-CD8) or not and were all orally challenged with 80 cysts from the 76K strain of T. gondii. Control mice were only infected. One month after oral challenge mice were killed. Spleen cells were cultured in the presence of parasite extract (10 µg/mL) and supernatants were collected between 24 and 96 h to determine cytokine levels (IL-4 and NO at 24 h, IL-2 at 48 h, IL-10 at 72 h and IFN-γ at 96 h). These results are representative of three independent experiments (*P < 0.05, **P < 0.01).

**Figure 2** Post-infection cellular response in spleen following CD8+ depletion. CBA/J mice were immunized twice with dendritic cells pulsed (TE-SRDC) or not (NP-SRDC) with T. gondii extract (TE). Fifteen days post-immunization, mice were depleted with a monoclonal antibody (α-CD8) or not and were all orally challenged with 80 cysts from the 76K strain of T. gondii. Control mice were only infected. One month after oral challenge mice were killed. Spleen cells were cultured in the presence of parasite extract (10 µg/mL) and supernatants were collected between 24 and 96 h to determine cytokine levels (IL-4 and NO at 24 h, IL-2 at 48 h, IL-10 at 72 h and IFN-γ at 96 h). Results shown are representative of three independent experiments (*P < 0.05, **P < 0.01).
could result from the decreased secretion of IFN-γ and/or IL-2. Indeed, IFN-γ is a well-known and powerful driver of IL-10 secretion. It could also be the case of a CD8⁺ T splenocytes secretion.

Nitric oxide production was significantly decreased in depleted groups. Indeed, NO concentrations in spleen cell supernatants were 12.3 μM for nondepleted NP-SRDC mice and 5.8 μM for CD8⁻-depleted mice (P < 0.05). In TE-SRDC mice, NO concentrations reached 14 μM for nondepleted mice and 8.8 μM for depleted mice (P < 0.05). As for IL-10, NO reduction could be a consequence of IFN-γ secretion decrease or CD8⁺ T lymphocytes could synthesize this molecule following DC vaccination and challenge in mice.

In MLNs (Figure 3), a small decrease in IFN-γ secretion was observed in depleted NP-SRDC mice (220 pg/mL; 332 pg/mL in nondepleted controls; not significant), whereas depleted TE-SRDC mice had significantly higher levels of IFN-γ secretion than TE-SRDC mice (8964 and 2391 pg/mL, respectively; P < 0.05). Likewise, TE-SRDC mice had a marked increase in IFN-γ secretion compared to the control group (2391 and 172 pg/mL, respectively; P < 0.05). Thus, CD8⁺ cells seem to exert a strong down-regulation of IFN-γ synthesis.

IL-2 secretion profile was similar to that observed for IFN-γ. Indeed, TE-SRDC mice produced significantly more IL-2 than the control group (2 and 0 pg/mL, respectively; P < 0.05) contrary to NP-SRDC mice (1.25 and 0 pg/mL, respectively; not significant). There was no significant difference between CD8⁻-depleted NP-SRDC mice and their control group (0.25 and 1.25 pg/mL, respectively). Finally, a significant increase in IL-2 secretion was observed following CD8⁻ depletion among TE-SRDC mice (6.75 and 2 pg/mL, respectively; P < 0.05). Thus as for IFN-γ, IL-2 would be down-regulated by CD8⁺ T lymphocytes.

IL-10 secretion was similar among most groups (57 pg/mL for control mice, 33 pg/mL for NP-SRDC mice, 51 pg/mL for depleted NP-SRDC mice and 109 pg/mL for TE-SRDC mice). Depleted TE-SRDC mice were the only ones to show a significant increase compared to their own control group (1132 and 109 pg/mL, respectively; P < 0.05). These results could either indicate a direct role for CD8⁺ cells in both Th1 and Th2 cytokine down-regulation in MLNs or an indirect effect of the CD8⁺ T cells depletion.

Nitric oxide production was not significantly different between either NP-SRDC mice and the control group (15.8 and 23.3 μM, respectively) or NP-SRDC and depleted NP-SRDC mice (15.8 and 13.2 μM, respectively). On the contrary, TE-SRDC mice and the control group had significantly different NO levels (9.5 and 23.3 μM, respectively; P < 0.05) as for depleted and nondepleted TE-SRDC mice (16.4 and 9.5 μM, respectively; P = 0.01). CD8⁺ lymphocytes are also implicated in NO down-regulation in MLNs. These results must be taken with care given that the concentrations of IL-2 are mainly under the sensitivity limit.

**Intracellular IFN-γ assay**

We tried to confirm the predominant role of CD8⁺ cells by quantifying the IFN-γ-producing CD4⁺ or CD8⁺ splenic T lymphocytes (Figure 4).

One month after their oral challenge, mice were sacrificed and CD4⁺ and CD8⁺ T cells were separated on microbeads. They were then cocultured with NP-SRDC or TE-SRDC before staining of intracellular IFN-γ and surface CD4 or CD8 molecules.

Analysis of IFN-γ positive CD8 T cells (Figure 4, upper panel) revealed no difference between CD4-depleted and nondepleted immunized mice (61 and 5.9% for NP-SRDC mice; 13.9 and 13.8% for TE-SRDC mice). Moreover,
there was an increased percentage of IFN-γ-positive in
TE-SRDC-immunized groups compared to NP-SRDC-
immunized groups (13.9 and 61%, respectively). These
data further indicate that CD8+ cells are the IFN-γ-secret-
ing cells induced following vaccination and challenge.

Analysis of CD4 T cells (Figure 4, lower panel) showed
that mice immunized with NP-SRDC and CD4-depleted
mice immunized with NP-SRDC or TE-SRDC had similar
percentages of IFN-γ-positive cells (1.8, 1.9 and 1.7%,
respectively). Mice immunized with TE-SRDC showed a
slight increase of IFN-γ-positive CD4 T cells (4.2%). Vac-
cinating mice with TE-SRDC could induce a small part of
the CD4+ population to secrete IFN-γ. Surprisingly, this
population gets back to a basal level when CD8+ cells are
depleted, reinforcing the major role played by these CD8+
cells.

**Effector function of splenic CD8+ T cells**

Consequently, we investigated whether CD8+ T cells stimu-
lated by TE-SRDCs immunization display specific cyto-
toxicity against target cells. Splenic CD8+ T lymphocytes
were cocultured with TE-sensitized SRDC cells or with
nonsensitized SRDC cells as a control, at various E/T
to ratios. Target cells and CD8+ T lymphocytes from mice
vaccinated with unpulsed SRDCs were used as controls.
CD8+ T lymphocytes obtained from mice vaccinated with
TE-pulsed SRDC demonstrated minimal levels of cyto-
toxicity, with <15% of SRDC cells undergoing cytolysis
(Figure 5a).

We next evaluated the IFN-γ and IL-2 releasing profile
of splenic CD8+ T cells following the immunization with
TE-pulsed-SRDC of CBA/J mice. IFN-γ and IL-2 profiles
were examined by ELISA. The levels of IFN-γ and IL-2
produced by the purified CD8+ T cells were three- and
sevenfold higher, respectively in TE-pulsed-SRDC–treated
CBA/J mice relative to unpulsed-SRDC of immunized
CBA/J mice (Figure 5b).

**Post-infection protection of CBA/J mice following CD4+ depletion**

Brain cysts were counted to evaluate protection of
depleted mice (Figure 6).

Mice immunized with NP-SRDC had a similar number
of cysts (6094 cysts/brain) to the control group (5875
cysts/brain). However, mice immunized with TE-SRDC
had a significantly lower parasitic load than controls
(2245 and 5875 cysts/brain, respectively; P < 0.01). This
decrease represented a protection level of 62%. CD4
depleted groups for both NP- and TE-SRDC mice tended
to have a slightly, but not significantly, higher number of
brain cysts than their respective control group (7659 and
6094 cysts/brain for NP-SRDC; 2595 and 2245
cysts/brain for TE-SRDC). Nevertheless, TE-SRDC mice
depleted of CD4+ cells still had a 56% protection level.
These data indicate that TE-SRDC vaccination protects
mice from cyst formation and that CD4+ T lymphocytes
play a minor role in this protection mechanism.

**Post-infection protection of CBA/J mice following CD8+ depletion**

CD8+-depleted NP-SRDC mice had a higher number of
brain cysts although the difference was not statistically
significant (7047 and 6094 cysts/brain, respectively)
(Figure 7). On the contrary, depleted TE-SRDC mice
had significantly more cysts than their nondepleted con-
trol group (4620 and 2245 cysts/brain, respectively;
P < 0.05). This finding suggests that CD8 depletion
results in a loss of protection following a vaccination
with pulsed DCs and an oral challenge (62% for nonde-
pleated TE-SRDC mice; 21% for CD8+-depleted TE-
SRDC mice).
DISCUSSION

Several previous studies suggest that DCs play an important role in the setting of the immune response to the intracellular parasite *T. gondii* during the early and chronic phases of infection. For instance, TE-pulsed DCs administered *in vivo* induce a strong humoral and cellular immune response and promote protection against a virulent challenge (13). Thus, DCs can effectively process *T. gondii* antigens for presentation *in vivo* but their use in a vaccine strategy is not acceptable. It is of interest to study the effector mechanisms induced by *T. gondii*-sensitized dendritic cells as a well-described protective immune response would help the development of new efficient vaccine strategies. This is why we investigated the relative contribution of the CD4+ or CD8+ T lymphocytes subpopulations in a model of chronically infected mice vaccinated with TE-pulsed DCs and depleted of these lymphocytes.

We chose to challenge our mice with cysts from the type II 76K strain of *T. gondii*, which is different from the parasite strain (RH, type I) used to pulse DCs. The protective immune responses could be improved by the use of *T. gondii* antigen from a type II strain (16). Johnson *et al.* demonstrated that splenocytes derived from C3H.L.d mice orally infected with the Me49 strain of *T. gondii* (type II)
have protective L^2-restricted CTL activity against target cells infected with Me49 tachyzoites but not against target cells infected with RH tachyzoites (type I). These results have implications for the development of a vaccine directed against type II T. gondii strains.

We first determined the role of CD4^+ T lymphocytes after an efficient depletion of over 90%. The results revealed a minor role for these cells since CD4-depleted or nondepleted mice have similar cytokine secretion profiles in spleen as well as in MLNs. Moreover, depleted mice did not show any significant loss of protection in terms of brain cyst load. These results contrast with those obtained by Casciotti et al. (9). They demonstrated that CD4^+ T cells are important for early IFN-\gamma production during T. gondii infection and that lack of CD4^+ lymphocytes leads to parasite multiplication in the tissues. Moreover, CD4-deficient mice exhibited parasite burdens in the brain. Johnson and Sayles (17) also showed the implication of CD4^+ cells as they induce CD8^+ T cells through the production of IL-2 and maintain CD8^+ T cell effector immunity. CD4^+ T lymphocytes also contributed significantly to protection against chronic infection via their role as helper cells for production of isotype-switched antibodies. The contradictory results obtained following infection alone or following both vaccination and infection could result from a particular orientation of the immune response. Indeed, in our protocol, DCs could directly prime CD8^+ T lymphocytes via cross-presentation of T. gondii antigens, as previously demonstrated by Gubbels et al. (18).

For this reason, we next studied the implication of CD8^+ T lymphocytes. In spleens CD8^+ cells seem to be responsible for cytokine synthesis. Indeed, their depletion leads to a significant decrease of both Th1 (IFN-\gamma and IL-2) and Th2 (IL-10 and IL-4) cytokines. The residual IFN-\gamma synthesis detected after CD8-depletion could be due to another cell type. For example, it has been demonstrated that splenic NK cells can produce this cytokine in response to chronic infection via their role as helper cells for production of isotype-switched antibodies. The contradictory results obtained following infection alone or following both vaccination and infection could result from a particular orientation of the immune response. Indeed, in our protocol, DCs could directly prime CD8^+ T lymphocytes via cross-presentation of T. gondii antigens, as previously demonstrated by Gubbels et al. (18).

In contrast to spleens, MLNs showed increased secretion of cytokines following CD8^+ depletion suggesting that CD8^+ T lymphocytes could act as regulatory cells. A recent review summarizes the current knowledge on CD8^+ Tregs, a newly described CD8^+ lymphocyte subtype with dedicated suppressor function (22). Although not proven in parasitic infections, their importance in autoimmunity is well-documented and they could be responsible for the moderation of the elicited immune response in local lymph nodes. It would be of importance to determine which cell population is responsible for the MLN IFN-\gamma secretion.

This is the first study to point to CD8^+ lymphocytes as the unique effector population responsible for the protection of mice following efficient DC vaccination and subsequent virulent challenge. This is partly in accordance with a previous description of CD8^+ T cells as effector lymphocytes while CD4^+ T cells were crucial for the regulation of the immune response in a very different vaccination assay (20).

Finally, we provide further insight into the long-term immunity that protects mice against T. gondii, a ubiquitous parasite resulting in severe sequelae in immunocompromised individuals. Future studies will be needed to determine how T. gondii antigens are presented to CD8^+ lymphocytes. A recent study showed encouraging results. Indeed, the authors demonstrated that CD8^+ DCs, like SRDCs are, were very efficient in processing and cross-presenting exogenous antigen to CD8^+ T cells. They also highlighted CD24 as an essential costimulatory molecule required for CD8^+ DCs to generate CD8^+ T-cell responses (23). The possible roles of various CD4^+ lymphocyte subtypes and other immune cell populations during the chronic phase of the disease also need to be elucidated, with a view in developing an effective vaccine to be used in animals that serve as a natural reservoir for human contamination.

A new vaccine strategy could be achieved by the direct in vivo targeting of endogenous dendritic cell populations to initiate the required immune response. Such protocol has already been successfully described. Chua et al. (24) showed that branched cationic peptides ligated to a cargo peptide consisting of a T-helper epitope and a CTL epitope from influenza virus were efficiently translocated into DCs and induced an antigen-specific CD8^+ T-cell response in the presence of maturation factors. In another viral model, Sabado et al. (25) described HIV uptake by DCs, probably via lectines, and subsequent processing via
TAP1/2 and loading onto MHC class I molecules in the endoplasmic reticulum. Finally, the next step to efficiently develop a vaccine strategy will be to identify which parasitic peptides are cross-presented by DCs to CD8+ T cells to initiate the specific protective response to *T. gondii*. Blanchard *et al.* (26) recently found that a decapeptide from the dense granule protein GRA6 could effectively induce such protection against *T. gondii*, as assessed by survival of mice. However, their study was conducted using bone marrow-derived DCs. It could be of interest to target in vivo splenic CD8+ DCs, known to protect our mice, with such putative protective parasitic peptides.

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