Improvement of a Dendritic Cell-Based Therapeutic Cancer Vaccine with Components of *Toxoplasma gondii*†

Masoumeh Motamedi,1 Samaneh Arab,2 Seied Mohammad Moazzeni,3 Masoumeh Khamis Abadi,2 and Jamshid Hadjati,2* Lorestan University of Medical Sciences, Khoramabad, Iran;1 Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran;2 and Department of Immunology, Tarbiat Modarres University, Tehran, Iran3

Received 18 May 2009/Returned for modification 21 May 2009/Accepted 28 July 2009

The use of dendritic cells (DCs) as a cellular adjuvant is a promising approach to the immunotherapy of cancer. It has previously been demonstrated that DCs pulsed ex vivo with *Toxoplasma gondii* antigens trigger a systemic Th1-biased specific immune response and induce protective and specific antitoxoplasma immunity. In the present study, we demonstrate that tumor antigen-pulsed DCs matured in the presence of *Toxoplasma gondii* components induce a potent antitumor response in a mouse model of fibrosarcoma. Bone-marrow derived DCs (BMDCs) were cultured in the presence of granulocyte-macrophage colony-stimulating factor and interleukin-4. After 5 days, tumor lysates with or without the *T. gondii* lysate were added to the culture for another 2 days. The cytokine production in the BMDC culture and the coculture supernatants of DCs and splenic cells was evaluated. For immunization, 7 days after tumor challenge, different groups of BALB/c mice received different kinds of DCs subcutaneously around the tumor site. Tumor growth was monitored, and 2 weeks after DC immunotherapy, the cytotoxic activity and the infiltration of CD8+ T cells were monitored in different groups. According to the findings, immunotherapy with *T. gondii*-matured DCs led to a significant increase in the activity of cytotoxic T cells and decreased the rate of growth of the tumor in immunized animals. Immature DCs did not cause any change in cytotoxic activity or the tumor growth rate compared to that in the healthy controls. The current study suggests that a specific antitumor immune response can be induced by DCs matured with *T. gondii* components and provide the basis for the use of *T. gondii* in DC-targeted clinical therapies.

Dendritic cells (DCs) efficiently induce T-cell activation in the secondary lymphoid organs (3, 4, 25). The Th1 arm of the immune response is very important in the battle against cancer (17). Evidence indicates that DCs play an important role in determining the type of immune response generated against antigens. Several factors can influence the development of polarized immune responses, such as the DC lineage and its activation status. Some studies have shown that distinct DC subsets are able to promote different types of responses, depending on the pathogen-derived signals and the host-derived cytokines present in the microenvironment (13, 23, 24, 37).

In microbial infections, certain molecular patterns of microbial components directly stimulate immature DCs in the periphery to differentiate into mature DCs by binding to pattern recognition receptors, such as Toll-like receptors (TLRs), which play a critical role in the innate immunity of mammals. The stimulation of TLR signaling in DCs causes an increase in the surface expression of the major histocompatibility complex (MHC) peptide for T-cell recognition, the upregulation of costimulatory molecules important for T-cell clonal expansion, and the secretion of immunomodulatory cytokines, which direct T-lymphocyte differentiation into effector cells. Remarkably, the ligation of distinct TLRs can trigger the production of different cytokines by a single DC type or result in different cytokines in distinct DC subtypes. Studying the complexity of the DC responses to TLR ligands illuminates the link between the innate and the adaptive arms of the immune system (1, 5, 36).

*Toxoplasma gondii* is an obligate intracellular parasite. Immunity to this organism is accomplished by the high-level production of type 1 cytokines such as gamma interferon (IFN-γ). Both interleukin-12 (IL-12) and IFN-γ are essential for resistance to this opportunistic pathogen (10, 12, 21, 31, 38). *T. gondii* is a potent stimulus for IL-12 production, which in turn is required to skew the immune response toward Th1 (16, 33). Innate immune cells, such as polymorphonuclear neutrophils, DCs, and macrophages, are important sources of IL-12 during *T. gondii* infection (7). TLR2 and myeloid differentiation factor 88 are critical for protective immunity against *T. gondii* infection (27). In recent years, TLR4 has also been found to be essential in the *T. gondii*-induced activation of DCs (2).

The present study was designed on the basis of the role of *T. gondii* in the induction of Th1 (cellular arm) immune responses and the importance of this arm in anticancer immunity. In the present study, the maturation state and the cytokine production capabilities of *T. gondii*-treated DCs, as well as their potential adjuvant effect on tumor immunotherapy of an experimental model, were evaluated.

**MATERIALS AND METHODS**

**Animals and cell line.** Female BALB/c mice were purchased from the Institute Pasteur of Iran. The mice were used at ages ranging from 6 to 8 weeks. All experiments with animals were performed according to the guidelines of the local ethical committee. BALB/c mouse-derived fibrosarcoma (WEHI 164) and colon carcinoma (CT26) cell lines were maintained by in vitro culture in RPMI 1640 (Sigma, Steinheim, Germany) supplemented with 10% heat-inactivated fetal bo-

* Corresponding author. Mailing address: Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran. Phone and fax: 98 2166419536. E-mail: hajatij@sina.tums.ac.ir.
† Published ahead of print on 5 August 2009.
washed, sonicated, and centrifuged. The protein concentration of the supernatant was measured by adding 1 mol/liter H2SO4 solution. The absorbance was measured at 450 nm with a reference wavelength at 690 nm by using an ELISA plate reader (Hype-rion Micro Reader 4 plus).

**Detection of cytokines by ELISA.** DC and T-cell cytokine production was detected in the supernatants of a DC culture (IL-12 p70) and a coculture supernatant of DCs and splenic cells (IL-10, IFN-γ). The supernatants were collected and kept frozen at −20°C. The cytokine concentrations were measured with an ELISA kit (BenderMed System, Austria), according to the manufacturer’s instructions.

**Immunization.** Seven days after tumor challenge, the mice were injected with 10°/200 μl phosphate-buffered saline (PBS) of BMDCs matured with T. gondii lysate (T. gondii lysate-DCs) or LPS (LPS-DCs) and immature DCs (IM-DCs) subcutaneously around the tumor site. The control group received PBS. Tumor measurements were performed every 2 days with calipers, which spanned the shortest and longest surface diameters. The mice were killed when the tumor diameter reached >400 mm².

**Cytotoxicity assay.** Two weeks after immunization the spleenocytes were isolated and used as effector cells. Cells of the WEHI 164 and CT26 tumor cell lines were used as target cells. Cytotoxic activity was measured with a lactate dehydrogenase (LDH) cytotoxicity detection kit (Roche Applied Science). After the effector and target cells were washed with assay medium (RPMI 1640 with 1% bovine serum albumin), the effector cells were cocultured with the target cells in a 96-well round-bottom plate for 6 h at 37°C. In some wells, target or effector cells were cultured alone to determine the amount of LDH spontaneously released by these cells. In some other wells containing target cells, 0.1 ml of 2% Triton X-100 lysis agent was added to measure the maximum amount of LDH releasable by target cells. The plates were then centrifuged, and the supernatants were transferred to another flat-bottom ELISA plate. The LDH detection mixture (0.1 ml) was added to each well and the plates were incubated for 30 min at room temperature. The absorbance at 490 nm was measured with an ELISA reader. The percentage of cell-mediated cytotoxicity was determined by the following equation: (experimental release – spontaneous target release – spontaneous effector cell release)/(maximal target release – spontaneous target release) × 100.

**Immunohistochemistry.** Tumors were resected 14 days after DC immunization and were embedded in optimum cutting temperature compound (Tissue Tek; Sakura Finetechical, Tokyo, Japan), and the embedded tumors were frozen in liquid nitrogen. Cryostat sections (5 μm) were thaw mounted on slides, air dried, and stored desiccated at −20°C. Sections were fixed for 2 min in cold acetone, hydrated in PBS, and incubated in protein-blocking solution (2% human albumin) for 15 min. Endogenous peroxidase activity was blocked with 0.3% H2O2. To detect murine CD8+ T cells, the sections were then incubated for 1 h with anti-CD8 MAB (clone 53.6.72; BD Biosciences). After the sections were washed in PBS, they were incubated with biotin-labeled anti-rat IgG and subjected to

---

**FIG. 1.** Phenotypic changes of BMDCs in response to LPS and T. gondii lysate (T.L). Day 5 immature and day 7 mature BMDCs were analyzed by flow cytometry for the expression of DC maturation markers. The data are presented as the percentages of positive cells for each marker and are representative of those from three separate experiments.

**FIG. 2.** Production of IL-12 by IM-DCs and after maturation with T. gondii lysate (T.L) and LPS. The cytokines in the supernatants of IM-DCs, T. gondii lysate-DCs, and LPS-DCs were quantified by an IL-12 (p70)–specific ELISA.
treatment with avidin-biotin-peroxidase complex by using a peroxidase kit (BD Biosciences). The color reaction was developed in 3,3-diaminobenzidine solution, and counterstaining was performed with Mayer’s hematoxylin solution. The number of CD8$^+$ cells per 10 high-power fields (magnification, ×400) was determined by light microscopy. The CD8-positive cells in each specimen were evaluated by two independent observers, and the percentage of these cells was obtained by dividing the number of stained cells by the total number of cells counted. The results were expressed as the mean ± standard error for each group.

Statistical analysis. The results are expressed as the means ± standard errors. Statistical analysis of all data except the survival data was performed by Student’s $t$ test. The survival data were analyzed by the Kaplan-Meier log-rank test. A $P$ value of <0.05 was considered significant. All calculations were performed with SPSS for Windows (version 10.0) software.

RESULTS

Effect of T. gondii lysate and LPS on DC maturation. To investigate the effect of the T. gondii lysate on the phenotype and maturation of DCs, BMDCs were cultured in the presence of the T. gondii lysate. The expression of MHC-II, CD40, CD80, and CD86 was analyzed by flow cytometry. About 74% of the DCs expressed the mouse-specific DC marker CD11c. T. gondii lysate-DCs expressed high levels of maturation compared to those for IM-DCs (Fig. 1).

IL-12 production by DCs. To determine the abilities of BMDCs to produce IL-12, the cytokine secreted in the supernatants of DC culture was quantified by ELISA. As shown in Fig. 2 a significant increase in the ability to produce IL-12 was detected in T. gondii lysate-DCs compared with that of LPS-DCs ($P < 0.001$) and IM-DCs ($P < 0.001$). The ability of T. gondii lysate-DCs and LPS-DCs to produce IL-12 was significantly higher than that of IM-DCs ($P < 0.001$).

Antigen-specific proliferation and cytokine production. To compare the abilities of DCs of different groups to support T-cell proliferation, MLR was performed with tumor antigen-primed mouse splenocytes as the responder cells. MLR was
performed by coculturing these cells with various numbers of irradiated DCs derived from each group of BALB/c mice for 5 days. In addition, the supernatants of day 2 cocultures were collected and assessed for their cytokine (IFN-γ, IL-10) contents. The cell proliferation of the responders was estimated by measurement of BrdU uptake by ELISA. Compared with both LPS-DCs (P < 0.001) and IM-DCs (P < 0.001), *T. gondii* lysate-DCs had a markedly increased capacity to generate MLR with BALB/c responder lymphocytes (Fig. 3a).

As shown in Fig. 3b, a significant increase in the level of IFN-γ production was detected in *T. gondii* lysate-DCs compared to the levels in LPS-DCs (P < 0.001) and IM-DCs (P < 0.001), whereas the level of production of IL-10 was lower (P < 0.5 and P < 0.001, respectively) (Fig. 3c). LPS-DCs also induced significantly higher levels of T-cell proliferation (P < 0.001) and IFN-γ production (P < 0.001) and lower levels of IL-10 production than IM-DCs (P < 0.001).

**CTL activity induced by intratumor injection of *T. gondii* lysate-DCs and LPS-DCs.** To confirm the induction of systemic and specific antitumor immunity, we examined the cytotoxic activities of T lymphocytes derived from the spleens of mice treated with DCs. Splenocytes were cultured with WEHI 164 or CT26 cells for 6 to 8 h, and the LDH release assay was performed. As shown in Fig. 4, the intratumor injection of *T. gondii* lysate-DCs induced significant cytotoxic T lymphocyte (CTL) activity against WEHI 164 cells compared with that induced by the other groups of DCs. Treatment with *T. gondii* lysate-DCs induced significantly higher levels of CTL activity (P < 0.001). A low level of CTL activity against CT26 cells was observed in all groups. These results suggest that the CTL activity induced by treatment with *T. gondii* lysate-DCs and LPS-DCs appeared to be specific for WEHI 164 tumor cells.

**Inhibition of tumor growth in vivo.** To examine the antitumor effects of DCs, $1 \times 10^6$ LPS-DCs, *T. gondii* lysate-DCs, or IM-DCs were injected around the established WEHI 164 cell tumors 7 days after tumor challenge. As shown in Fig. 5a, the *T. gondii* lysate-DCs had the most suppressive effect on tumor growth. The *T. gondii* lysate-DCs and LPS-DCs suppressed the growth of tumors more significantly than the IM-DCs (P = 0.007 and P = 0.043, respectively).

The 26-day rates of survival after tumor inoculation for mice treated with *T. gondii* lysate-DCs, LPS-DCs, IM-DCs, and the control group were 100, 100, 20, and 0%, respectively (Fig. 5b). The log-rank test revealed that treatment with *T. gondii* lysate-DCs and LPS-DCs significantly prolonged survival compared
to the length of survival achieved with IM-DCs and PBS (control treatment) ($P < 0.05$).

**Tumor infiltration of CD8$^+$ T cells.** The CD8$^+$ T-cell infiltration into tumor cells in different groups of mice immunized with DCs was determined by an immunohistochemical method. The infiltration of a larger number of CD8$^+$ T cells was shown in mice receiving $T. gondii$ lysate-DCs and LPS-DCs than in mice in the control groups. As shown in Fig. 6, the percent CD8$^+$ cell infiltration was determined for all groups. The level of infiltration of CD8$^+$ T cells in the $T. gondii$ lysate-DC group was 41% and that in the LPS-DC group was about 39%, whereas it was about 1% in the IM-DC and PBS groups.

**DISCUSSION**

Previous studies have revealed that DCs exhibit different activation responses to different classes of pathogens in vitro and that there appears to be a correlation between the activation status of DCs and the type of T-helper cell response that they are able to induce (26). However, DCs are used extensively for antigen-specific immunotherapy of cancer because they are the only antigen-presenting cells capable of inducing the initial immune response (15).

$T. gondii$ is an intracellular protozoan that induces a strong Th1 response during acute infection; the development of this response and the production of IFN-γ are required for host survival during both the acute and the chronic stages of Toxoplasma infection (11). In this study, we analyzed the effects of $T. gondii$ cellular components on DC maturation, cytokine production, and T-cell activation in vitro and their efficacies as maturation factors for DCs for use as immunotherapy for experimental model of tumors in vivo.

DCs control T-cell responses by the production of cytokines. DC-derived IL-12 is the major Th1-driving factor (20, 34). In the present study, we show that $T. gondii$-matured DCs produce large amounts of IL-12 and small amounts of IL-10. Splenic DCs in mice infected with $T. gondii$ became highly activated, and infection with $T. gondii$ resulted in dramatically increased levels of expression of activation markers and elevated levels of IL-12 production by DCs. In comparison with LPS, used as the standard maturation factor for DCs (14), $T. gondii$ significantly enhanced the production of IL-12. The IFN-γ secreted by T cells can exert direct antiproliferative and antimetabolic effects on a wide variety of tumor cells, while IL-10 inhibits the stimulatory capacity of DCs and impairs DC-mediated Th1 cell differentiation, promoting the Th2 phenotype and T-cell anergy (8, 9). In this study, T cells cocultured with $T. gondii$-matured DCs produced large amounts of IFN-γ. $T. gondii$ lysate-DCs consistently caused a significant decrease in the level of IL-10 production of T cells compared to that induced by IM-DCs. Therefore, $T. gondii$ can stimulate DCs to shift the immune response toward the type 1 response. According to the findings of previous studies, live but not killed tachyzoites of $T. gondii$ upregulate CD40, CD80, CD86, and MHC-II molecules on human DCs and activated DCs induce the production of large amounts of IFN-γ by T cells (35).

Mature DCs are specialized in the activation of T cells because of the high level of expression of MHC and costimulatory molecules (3). In the present study, $T. gondii$ increased the levels of expression of MHC-I, MHC-II, CD80, CD86, and CD40 molecules on human DCs and activated DCs induce the production of large amounts of IFN-γ by T cells (35).

Activation of DCs is crucial for the priming of CTLs, which have a critical role in tumor immunity (29, 32). In the cytotoxicity assay, we showed that a DC vaccine generated in the presence of $T. gondii$ induced a strong specific CTL response against our murine fibrosarcoma tumor model. The immunohistochemical study also showed that the level of CD8$^+$ T-cell infiltration of the tumor in $T. gondii$ lysate-DC-treated animals...
was significantly higher than that in animals treated with LPS-DCs, IM-DCs, and PBS (controls). Our data indicate that vaccinations with \textit{T. gondii}-stimulated DCs are even more protective and inhibit tumor growth more than LPS-stimulated DCs.

In this study we did not characterize the exact component of \textit{Toxoplasma} which caused a significant effect on DCs. However according to the findings from different investigations, the initiation of immune responses by DCs is promoted by various exogenous or endogenous stimuli, including LPS, tumor necrosis factor alpha, and heat shock protein (6, 19, 30). It was demonstrated that \textit{T. gondii} heat shock protein 70 is capable of inducing the phenotypic and functional differentiation and maturation of murine BMDCs, and this effect is dependent on TLR4 (2).

In summary, in this study, we demonstrated that the administration of \textit{T. gondii}-matured DCs as a therapeutic vaccine is able to induce a pronounced CTL-mediated antitumor immune response which leads to retarded tumor growth and the prolonged survival of the tumor-bearing mice. This approach is applicable to the generation of DC-based antitumor vaccines which could be evaluated in clinical trials as a treatment for appropriate human cancers.

**ACKNOWLEDGMENTS**

We are grateful to Ahmad Jalili of the Medical University of Vienna for helpful comments. This study was supported by a grant 132/10601 from the Tehran University of Medical Sciences.

**REFERENCES**

3. Akira, S. 2001. Endo-

**Downloaded from http://cvl.asm.org/ on November 6, 2017 by guest**