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Human Dendritic Cells Discriminate Between Viable and Killed *Toxoplasma gondii* Tachyzoites: Dendritic Cell Activation After Infection with Viable Parasites Results in CD28 and CD40 Ligand Signaling That Controls IL-12-Dependent and -Independent T Cell Production of IFN-γ[1]

Carlos S. Subauste[2] and Matthew Wessendarp

We studied how the interaction between human dendritic cells (DC) and *Toxoplasma gondii* influences the generation of cell-mediated immunity against the parasite. We demonstrate that viable, but not killed, tachyzoites of *T. gondii* altered the phenotype of immature DC. DC infected with viable parasites up-regulated the expression of CD40, CD80, CD86, and HLA-DR and down-regulated expression of CD115. These changes are indicative of DC activation induced by *T. gondii*. Viable and killed tachyzoites had contrasting effects on cytokine production. DC infected with viable *T. gondii* rather than DC that phagocytosed killed parasites induced secretion of high amounts of IFN-γ by T cells from *T. gondii*-seronegative donors. IFN-γ production in response to DC infected with viable parasites required CD28 and CD40 ligand (CD40L) signaling. In addition, this IFN-γ response was dependent in part on IL-12 secretion. Production of IL-12 p70 occurred after interaction between T cells and DC infected with viable *T. gondii*, but not after incubation of T cells with DC plus killed tachyzoites. IL-12 synthesis was inhibited by blockade of CD40L signaling. IL-12-independent IFN-γ production required CD80/CD86-CD28 interaction and, to a lesser extent, CD40-CD40L signaling. Taken together, *T. gondii*-induced activation of human DC is associated with T cell production of IFN-γ through CD40-CD40L-dependent release of IL-12 and through CD80/CD86-CD28 and CD40-CD40L signaling that mediate IFN-γ secretion even in the absence of bioactive IL-12. *The Journal of Immunology,* 2000, 165: 1498–1505.

Cell-mediated immunity that results in IL-12 and IFN-γ secretion is required for control of intracellular pathogens (1). Identification of the events that regulate cytokine production during infection with these organisms is crucial to our understanding of the mechanisms that determine whether protective immunity is elicited. APC should receive special attention, since events pivotal to the induction of protection against intracellular pathogens are those that transpire during the interaction between APC and T cells. In this regard, we have demonstrated that CD28-CD80/CD86 signaling regulates IFN-γ production, and CD40 ligand (CD40L)[3]-CD40 interaction regulates IL-12 and IFN-γ secretion during the cross-talk between T cells and monocytes infected with the intracellular protozoan *Toxoplasma gondii* (2, 3).

Dendritic cells (DC) are considered the APC responsible for the generation of primary immune responses (4). DC originate in the bone marrow and reach peripheral tissues through the blood. After encountering Ags and in response to inflammatory mediators, DC undergo a maturation process characterized by increased expression of MHC and costimulatory molecules (5–8). These changes are accompanied by migration to T cell-dependent areas of secondary lymphoid organs where mature DC stimulate naive T cells (5, 9, 10). However, there is less information regarding how this process influences the generation of cell-mediated immunity against intracellular pathogens.

We have demonstrated that unprimed human T cells secrete IFN-γ in response to *T. gondii*-infected APC (2, 3, 11). Therefore, the in vitro model of *T. gondii* infection is well suited to study how the interaction between DC and an intracellular pathogen affects the generation of T cell-mediated immunity. We demonstrate that live, but not killed, tachyzoites of *T. gondii* up-regulate CD40, CD80, CD86, and MHC class II molecules on human DC. In turn, DC activation is associated with the production of high amounts of IFN-γ by T cells from *T. gondii*-seronegative donors through CD40-CD40L-dependent IL-12 secretion and through CD80/CD86-CD28 and CD40-CD40L interactions that act via a mechanism that does not require IL-12.

Materials and Methods

Abs and cytokines

The following mAbs were used for cell purifications: anti-CD2, anti-CD3, anti-CD8, anti-CD56 (all from Becton Dickinson, San Jose, CA), anti-CD11b (OKM1, American Type Culture Collection, Manassas, VA), anti-

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Division of Infectious Diseases, University of Cincinnati College of Medicine, Cincinnati, OH 45267

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[2] Address correspondence and reprint requests to Dr. Carlos S. Subauste, Division of Infectious Diseases, Department of Medicine, University of Cincinnati College of Medicine, P.O. Box 670560, Cincinnati, OH 45267-0560. E-mail address: carlos.subauste@uc.edu

[3] Abbreviations used in this paper: CD40L, CD40 ligand; b-DC, blood dendritic cell; CM, complete medium; MFI, mean fluorescence intensity; DC, dendritic cell; md-DC, monocyte-derived DC; TLA, *T. gondii* lysate Ags.

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CD16 (Medarex, Annandale, NJ), anti-CD19 (Coulter, Hialeah, FL), and anti-glycophorin A (10F7 MN, gift from Rene de Waal Malefyt, DNAX Research Institute, Palo Alto, CA).

CTLA-4-Ig (gift from Bristol-Myers Squibb, Princeton, NJ) (12) and mAbs against CD40L (M90, gift from Immunex, Seattle, WA) were used in functional assays (all at 10 μg/ml). Isotype-matched mAbs and human IgG were obtained from PharMingen (San Diego, CA) and Sigma (St. Louis, MO), respectively. GM-CSF and IL-4 were purchased from Pepro-Tech (Rocky Hill, NJ).

The following conjugated or unconjugated mAbs were used for flow cytometry (purchased from Becton Dickinson, except when indicated): FITC-anti-CD3, FITC-anti-CD14, FITC-anti-CD19 (Caltag, South San Francisco, CA), FITC-anti-CD40 (PharMingen), FITC-anti-CD56, FITC-anti-HLA-DR (Caltag), PE-anti-CD80, PE-anti-CD86 (PharMingen), PE-anti-HLA-DR (Caltag), unconjugated anti-CD80, unconjugated anti-CD83 (HB-15a, gift from Thomas Tedder, Duke University, Durham, NC) (13), and unconjugated rat anti-CD115 (M-CSF receptor, 2-4A5, Zymed, San Francisco, CA). FITC-F(ab′)$_2$ rabbit anti-mouse IgG (Serotec, Oxford, U.K.) and cyanin-5-F(ab′)$_2$ goat anti-rat IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA) were used as secondary Ab when necessary. Unrelated murine mAbs (PharMingen) and rat IgG2a (Zymed) were used as negative controls.

Cell purifications

Using centrifugation on Ficoll-Hypaque gradients (Pharmacia LKB Biotechnology, Piscataway, NJ), PBMC were isolated from buffy coats of heparinized blood of healthy volunteers donors obtained from the Hoxworth Blood Center (Cincinnati, OH). Serologic tests for detection of anti-*T. gondii* IgG and IgM were performed in all samples of blood. Unless otherwise sated, the samples used had no demonstrable *T. gondii* IgG or IgM Abs.

Monocyte-derived DC (md-DC) were obtained as described previously (14, 15). Briefly, purified monocytes (1×10^6/ml) isolated as previously described (2) were incubated in complete medium (CM) consisting of RPMI 1640 with 10% FBS (HyClone, Logan, UT) that contained 1000 U/ml GM-CSF and 500 U/ml IL-4. Cytokines were replenished every 3–4 days. Cells were used after 7 days of in vitro culture.

Blood DC (b-DC) were obtained following a modification of a previously described protocol (16). PBMC were incubated with neuraminidase-treated SRBC. Nonrosetting cells were treated with anti-CD3, anti-CD8, anti-CD11b, anti-CD16, anti-CD19, and anti-glycophorin A mAb followed by incubation with magnetic beads coated with anti-mouse IgG (PerSeptive Biosystems, Framingham, MA). Populations obtained after removal of rosetting cells with a magnet contained >90% b-DC as defined by previously established criteria (HLA-DR$^+$, CD3$^-$ CD14$^{-/low}$ CD19$^-$ CD56$^-$ cells) (16) and contained <4% monocytes (CD14^{high}). Resting T cells (>99% CD3$^+$) were obtained as previously described (2). Cells were cultured in RPMI 1640 with 10% dye test-negative human AB serum (Gemini Bio-Products, Calabasas, CA). b-DC were not used in experiments that addressed *T. gondii*-induced DC activation and the effects of different parasite preparations on cytokine secretion, because b-DC spontaneously undergo maturation in vitro.

T. gondii *and infection*

Tachyzoites of the RH strain were obtained from infected monolayers of human foreskin fibroblasts as well as from peritoneal fluid of mice (17). DC were infected with *T. gondii* and cultured in Teflon vessels as previously described (2). The dose of tachyzoites per DC was 0.5/1 when studying cytokine production and 2/1 for phenotypic analysis. Neither uninfected human foreskin fibroblasts nor tachyzoite-free peritoneal lavage fluids from infected mice (after passage through a 0.45-μm pore size filter) mediated changes in the expression of the surface molecules tested. In certain experiments tachyzoites were killed by incubation in 1% paraformaldehyde in PBS (17). *T. gondii* lysate Ag (TLA) was prepared as described previously (2) and used at 10 μg/ml. Antigenic preparations were devoid of detectable levels of endotoxin (<0.015 EU/ml) using a *Limulus* amebocyte lysate assay (Sigma). The percentage of cells with intracellular tachyzoites was determined by light microscopy (2).

Flow cytometry

Cells were incubated for 30 min with 250 μg/ml human IgG (Sigma) to block Fc receptors. This was followed by 30-min incubation at 4°C with unconjugated mAb or isotype control Ab in PBS containing 1% FBS and 0.1% sodium azide. Cells were washed and counterstained with the appropriate conjugated secondary Ab. After blocking with mouse IgG, cells were stained with conjugated mAbs. Cells were fixed in 1% paraformaldehyde and analyzed using a FACSCalibur (Becton Dickinson). Corrected mean fluorescence intensity (MFI) was calculated by subtracting the MFI of the appropriate isotype control mAb from the MFI of each specific mAb. Sorting of DC into CD115$^-$ CD86^{high} and CD115$^+$ CD86^{int} was performed after 18-h incubation with *T. gondii*.

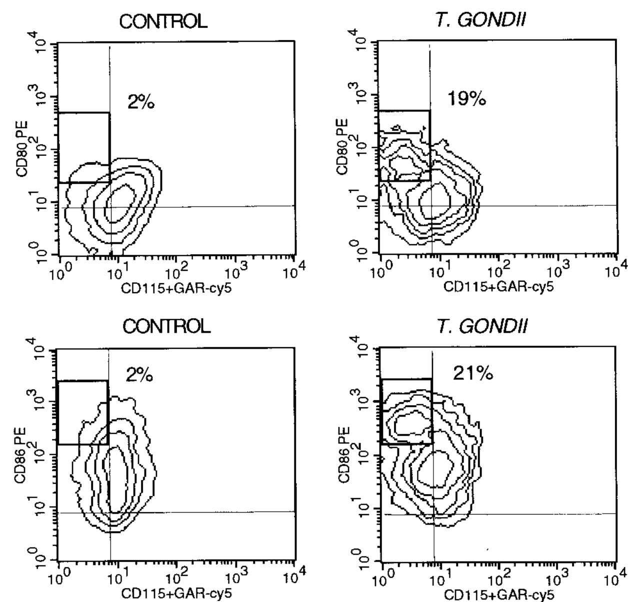

FIGURE 1. *T. gondii* up-regulates DC expression of CD80 and CD86. After 48 h of in vitro culture, md-DC were subjected to two-color FACS analysis using anti-CD115 mAb plus cyanin-5-goat anti-rat (GAR) and either PE-anti-CD80 or PE-anti-CD86 mAbs. The box within the contour graph and the percentage indicate either CD115$^-$ CD80^{high} or CD115$^-$ CD86^{high} DC. The results of one representative experiment of 10 are shown.

Cytokine assays

Purified resting peripheral blood T cells were incubated in 96-well plates with either uninfected or *T. gondii*-infected DC. Concentrations of T cells and DC were 1×10^6/ml and 2.5×10^5/ml, respectively, when studying IFN-γ production and 2×10^6/ml and 5×10^5/ml, respectively, for assays of IL-12 secretion. Abs were added to DC 30 min before incubation with T cells. Concentrations of IL-12 (p40 or p70; R&D Systems, Minneapolis, MN) and IFN-γ (Endogen, Cambridge, MA) were measured by ELISA in supernatants collected at 24 and 72 h, respectively. The lower limit of detection was 39 pg/ml for IFN-γ and IL-12 p40, and 0.6 pg/ml for IL-12 p70. The data in the figures are presented as the mean of triplicate wells ± SEM. In addition, the percent inhibition of cytokine production was calculated in each experiment that examined the effects of neutralizing Ab. The mean percent inhibition ± SEM of all comparable experiments are shown in the text.

Statistical analysis

Statistical significance was assessed by ANOVA and Student's *t* test.

Results

Viable, but not killed, T. gondii *tachyzoites activate human DC*

DC generated after culturing monocytes with GM-CSF and IL-4 (md-DC) exhibit a phenotype characteristic of immature DC (CD115$^+$ CD80^{low} CD86^{int} CD83$^-$) (18, 19). These cells were used to determine whether *T. gondii* alters the phenotype of human DC. Incubation with viable tachyzoites resulted in the appearance of a subpopulation of md-DC that exhibited down-regulation of the macrophage marker CD115 (Fig. 1). Loss of CD115 expression was accompanied by up-regulation of CD80 and CD86. On the average, CD80^{high} CD115$^-$ and CD86^{high} CD115$^-$ md-DC had 2.6 ± 0.4 times higher expression of CD80 (MFI, 42.4 ± 4.3 vs 17.4 ± 3.5) and 1.7 ± 0.2 times higher expression of CD86 (MFI,

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Table I. *Viable* T. gondii *up-regulates CD40 and HLA-DR on md-DC*[a]

	cMFI			
	Control	Killed *T. gondii*	Viable *T. gondii* $CD115^-$ $CD86^{high}$	Fold Increase (cMFI)
CD40	21.1 ± 3.6	18.7 ± 2.5	30.6 ± 4.2	1.4 ± 0.1
HLA-DR	191.0 ± 15.9	162.4 ± 10.2	420.4 ± 59.7	2.2 ± 0.3

[a] md-DC were incubated for 48 h with either killed or viable tachyzoites of *T. gondii* or with CM alone (Control). Thereafter, DC were subjected to three-color FACS analysis using anti-CD115 followed by goat anti-rat Cy5, plus PE anti-CD86 and either FITC anti-CD40 or FITC anti-HLA-DR mAbs. Increase in cMFI refers to CD40 and HLA-DR expression on $CD115^-$ $CD86^{high}$ DC induced by *T. gondii* compared to control md-DC. Killed *T. gondii* failed to alter DC phenotype even when using a concentration of parasites 2-fold higher than that of viable tachyzoites. Results represent the pool of seven individual experiments.

576.4 ± 32.3 vs 335.3 ± 56.9) than control md-DC (n = 10). Simultaneous staining with anti-CD80, anti-CD86, and anti-CD115 mAbs indicated that $CD80^{high}$ $CD115^-$ were also $CD86^{high}$ (data not shown).

Because CD40 and HLA-DR are required for optimal T cell responses against *T. gondii* (3, 11), we analyzed the expression of these surface molecules on md-DC incubated with or without *T. gondii*. To this end, DC were stained with anti-CD115 plus anti-CD86 mAbs and either anti-CD40 or anti-HLA-DR mAbs. As shown in Table I, $CD115^-$ $CD86^{high}$ md-DC induced by incubation with viable tachyzoites up-regulated HLA-DR and CD40 (n = 7).

Next, we studied whether viability of tachyzoites was required for the induction of phenotypic changes on DC. In contrast to results obtained with viable tachyzoites, incubation of md-DC with killed *T. gondii* failed to alter the expression of CD80, CD86, and CD115 (Fig. 2). Similarly, killed tachyzoites did not up-regulate DC expression of CD40 and HLA-DR (Table I). These results were not caused by an inability of md-DC to internalize killed tachyzoites, because the percentages of DC with intracellular tachyzoites after 2-h incubation with either viable or killed parasites were 40 ± 4 and 24 ± 1%, respectively (n = 3). Increasing the dose of killed *T. gondii* 2-fold failed to alter the md-DC phenotype. Therefore, the changes in expression of these surface molecules appeared unlikely to be due simply to phagocytosis of *T. gondii*. Taken together, incubation with viable, but not killed, tachyzoites induces DC activation.

DC maturation is associated with induction of CD83 (13, 19, 20). Therefore, md-DC were stained with anti-CD83, anti-CD86, and anti-CD115 mAbs to determine whether *T. gondii* up-regulates

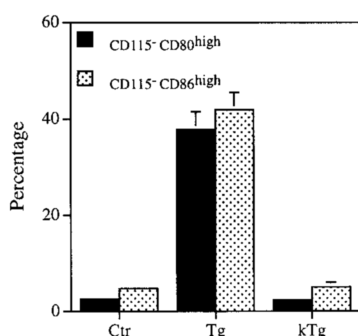

FIGURE 2. Viable, but not killed, *T. gondii* tachyzoites up-regulate CD80 and CD86 on DC. The md-DC were incubated with viable tachyzoites of *T. gondii* (Tg), killed *T. gondii* (kTg), or CM alone (Control). The percentage of $CD115^-$ $CD80^{high}$ and $CD115^-$ $CD86^{high}$ md-DC were determined by flow cytometry. Killed *T. gondii* failed to alter DC phenotype even when using a concentration of parasites 2-fold higher than that of viable tachyzoites. The results shown represent data pooled from four independent experiments.

CD83. Although *T. gondii*-induced $CD115^-$ $CD86^{high}$ md-DC could acquire CD83 expression (19.3 ± 3.7% $CD83^+$ compared with 1.9 ± 0.4% $CD83^+$ cells among $CD115^+$ $CD86^{int}$ DC), induction of CD83 occurred in only three of six independent experiments (data not shown).

Experiments were conducted to further explore the role of *T. gondii* infection on DC activation. The md-DC incubated with viable *T. gondii* were sorted by FACS into $CD115^-$ $CD86^{high}$ and $CD115^+$ $CD86^{int}$ cells. Microscopic examination of these cells revealed that whereas 51 ± 0.7% of $CD86^{high}$ $CD115^-$ md-DC had intracellular tachyzoites, only 1 ± 0.2% of $CD86^{int}$ $CD115^+$ md-DC contained intracellular *T. gondii* (n = 3). Thus, infection with viable *T. gondii* leads to DC activation.

Infection of DC with viable tachyzoites is required to trigger optimal IFN-γ production by T cells from T. gondii*-seronegative individuals*

We studied whether the contrasting effects of viable and killed parasite preparations on DC activation were associated with differences in cytokine production. Whereas T cells from *T. gondii*-seronegative individuals secreted high amounts of IFN-γ when incubated with DC infected with viable *T. gondii*, stimulation of T cells with DC plus killed tachyzoites resulted in the production of markedly lower concentrations of IFN-γ (Fig. 3*A*, 2,847 ± 1,042 vs 331 ± 142 pg/ml, respectively; $p < 0.01$; n = 7). These differences in IFN-γ production were not caused by dissimilarities in the percentages of DC with intracellular tachyzoites. The aforementioned experiments as well as all studies of cytokine production described below were conducted with a concentration of killed parasites that was 2-fold higher than that of viable tachyzoites. Thus, the percentages of DC with intracellular tachyzoites were 5.5 ± 0.4 and 4.7 ± 0.2 for DC incubated with viable or killed *T. gondii*, respectively. Moreover, the marked differences in IFN-γ secretion did not appear to be due to an inability of T cells to recognize DC plus killed parasites, since in the presence of exogenous IL-12, T cells stimulated with DC plus either viable or killed tachyzoites produced high amounts of IFN-γ (Fig. 3*B*). Finally, in contrast to T cells from *T. gondii*-seronegative donors, T cells from healthy individuals chronically infected with *T. gondii* secreted high amounts of IFN-γ in response to DC plus either viable or killed tachyzoites (13,373 ± 1,202 and 11,962 ± 868 pg/ml, respectively; n = 3; data not shown).

IL-12 p70 is secreted only after interaction between T cells and DC infected with viable T. gondii *tachyzoites*

The studies shown in Fig. 3*B* raised the possibility that differences in IFN-γ secretion after T cells were stimulated with DC plus either viable or killed tachyzoites might be caused by differences in the production of bioactive IL-12. To begin to explore this possibility, we studied the effects of a neutralizing anti-IL-12 mAb on

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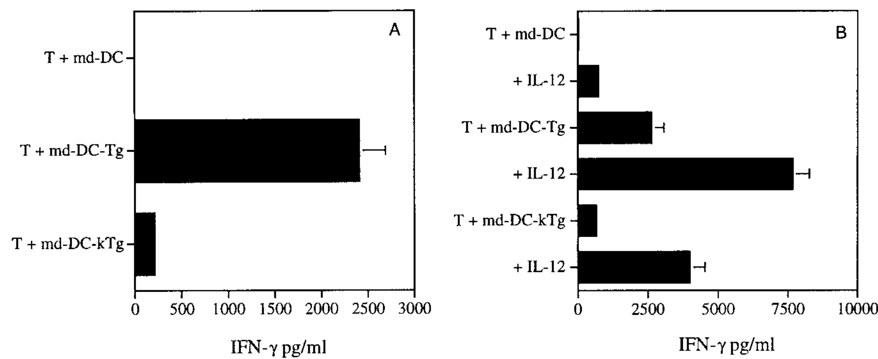

FIGURE 3. T cells secrete optimal amounts of IFN-γ after stimulation with DC incubated with viable, but not killed, *T. gondii* tachyzoites. *A*, Purified resting T cells (1×10^6/ml) from *T. gondii*-seronegative donors were incubated with md-DC (2.5×10^5/ml) with or without viable (Tg) or killed (kTg) tachyzoites of *T. gondii*. The concentration of killed *T. gondii* was twice that of viable *T. gondii*, so that percentages of DC with intracellular tachyzoites were equivalent in both groups. *B*, T cells and md-DC were cultured as described in *A* with or without rIL-12 (2.5 ng/ml). Supernatants were collected after 72 h and were used to measure concentrations of IFN-γ by ELISA. The results shown represent one of three (*B*) to seven (*A*) independent experiments.

IFN-γ production. Fig. 4 shows that whereas anti-IL-12 mAb induced a significant inhibition of IFN-γ secretion in response to md-DC infected with viable *T. gondii* (55.9 ± 2.0% inhibition; $p < 0.01$; $n = 12$; Fig. 4*A*), in parallel experiments this mAb did not affect the low level IFN-γ production triggered by md-DC and killed tachyzoites (Fig. 4*B*). At the concentration of mAb used in these studies, anti-IL-12 mAb neutralized induction of IFN-γ secretion mediated by addition of 1 ng of rIL-12 to T cells stimulated with either md-DC plus killed tachyzoites or monocytes plus TLA (94.8 ± 1.2% inhibition; $p < 0.0001$; $n = 3$; Fig. 4*C*). The lack of effect of anti-IL-12 mAb on IFN-γ production caused by md-DC plus killed parasites was not due to a general inability to modulate cytokine secretion, because blockade of the CD28 and CD40L pathways abrogated IFN-γ secretion (Fig. 4*B*). Thus, whereas IFN-γ production in response to DC plus viable *T. gondii* is partially dependent on IL-12 secretion, IFN-γ production triggered by DC plus killed tachyzoites is largely independent of bioactive IL-12 production.

Next, we determined whether incubations with viable and killed tachyzoites have differential effects on the secretion of bioactive IL-12. Stimulation of T cells with md-DC infected with viable *T. gondii* resulted in IL-12 p70 production (Fig. 5). In contrast, no IL-12 p70 was detected after T cell stimulation with md-DC incubated either alone or with killed *T. gondii*. IL-12 p70 production required T cells, because md-DC incubated with viable parasites failed to release IL-12 p70 in the absence of T cells. Taken together, interaction between T cells and md-DC infected with viable *T. gondii* is required for IL-12 p70 secretion.

CD28 and CD40L signaling regulate IFN-γ production triggered by DC and T. gondii

We have previously demonstrated that the CD28 and CD40L pathways are crucial for IFN-γ secretion by presumably unprimed T cells stimulated with *T. gondii*-infected monocytes (2, 3). The results described above indicate that T cell-APC cognate interaction also regulates IFN-γ production by T cells stimulated with DC plus killed *T. gondii*. Therefore, we determined whether CD28 and CD40L signaling control IFN-γ production in response to DC infected with viable *T. gondii*. As shown in Fig. 6*A*, IFN-γ secretion by T cells from *T. gondii*-seronegative individuals was significantly inhibited by either anti-CD40L mAb (56.4 ± 3.1% inhibition; $p < 0.03$; $n = 5$), or CTLA-4-Ig (63.0 ± 7.2% inhibition; $p < 0.03$; $n = 5$). Moreover, simultaneous addition of these two molecules resulted in further inhibition of IFN-γ secretion (83.5 ± 4.2% inhibition; $p < 0.01$; $n = 5$).

To confirm the relevance of CD28 and CD40L signaling for regulation of IFN-γ secretion, experiments similar to those described above were performed using b-DC. Fig. 6*B* shows that IFN-γ production in response to *T. gondii*-infected b-DC was significantly inhibited by either anti-CD40L mAb (52.3 ± 6.4% inhibition; $p < 0.03$; $n = 5$) or CTLA-4-Ig (69.1 ± 6.1% inhibition; $p < 0.01$; $n = 5$). Combination of these two molecules resulted in

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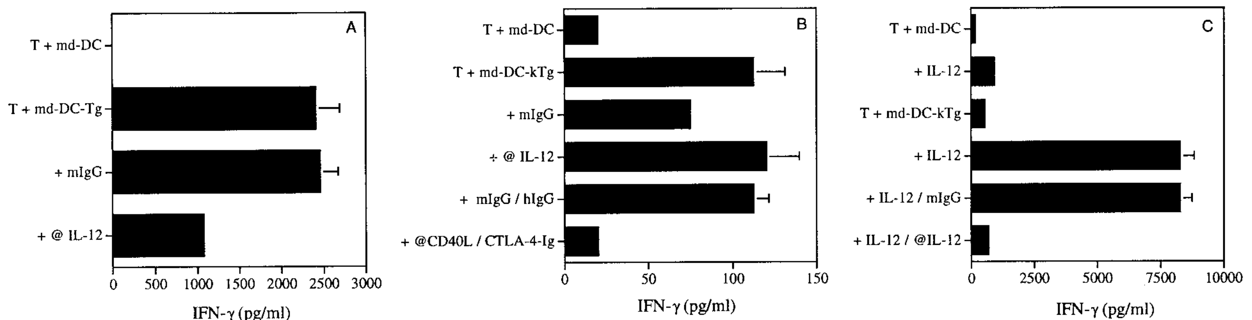

FIGURE 4. T cell production of IFN-γ in response to DC infected with viable *T. gondii* is partially dependent on IL-12 secretion. *A*, T cells (1×10^6/ml) were incubated with md-DC (2.5×10^5/ml) that were either uninfected or infected with viable *T. gondii*. Neutralizing anti-IL-12 mAb or isotype control mAb were used at 10 μg/ml. *B*, T cells were incubated with uninfected md-DC or md-DC that phagocytosed killed *T. gondii* (kTg) in the presence of anti-IL-12 mAb, anti-CD40L mAb, CTLA-4-Ig, or mouse or human IgG. All Ab were used at 10 μg/ml. Samples with undetectable IFN-γ are shown as having a cytokine concentration half the lower limit of detection of the ELISA. *C*, T cells were incubated with md-DC plus killed *T. gondii* with or without rIL-12 (1 ng/ml) in the presence of anti-IL-12 or isotype control mAbs. The results of one representative experiment of three (*B* and *C*) or 12 (*A*) are shown.

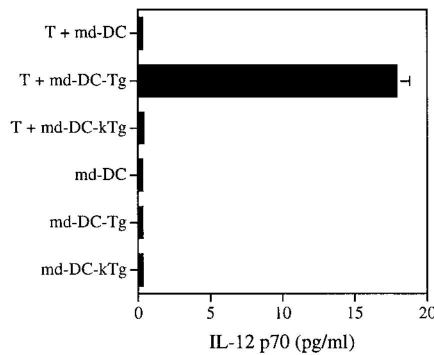

FIGURE 5. IL-12 p70 is secreted during interaction between T cells and DC infected with viable *T. gondii*. The md-DC (5×10^5/ml)) incubated with or without viable (Tg) or killed tachyzoites (kTg) of *T. gondii* were cultured in the presence or the absence of T cells (2×10^6/ml). Supernatants were collected after 24 h and were used to measure concentrations of IL-12 p70 by ELISA. Samples with undetectable IL-12 p70 are shown as having a cytokine concentration half the lower limit of detection of the ELISA. The results shown represent one of three independent experiments.

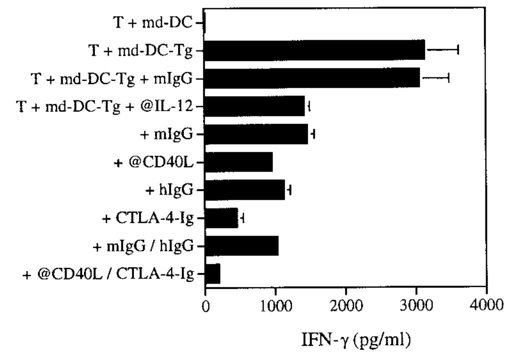

FIGURE 7. The CD80/CD86-CD28 and CD40-CD40L pathways regulate IL-12-independent T cell production of IFN-γ in response to *T. gondii*-infected DC. T cells (1×10^6/ml) were stimulated with either uninfected or *T. gondii*-infected md-DC (2.5×10^5/ml). Anti-IL-12, anti-CD40L mAbs, CTLA-4-Ig, and mouse or human IgG were added as indicated. The results shown represent one of four independent experiments.

further inhibition of IFN-γ secretion (80.5 ± 5.6% inhibition; $p < 0.01$; $n = 5$). In addition, similar to md-DC, IFN-γ production in response to *T. gondii*-infected b-DC was significantly inhibited by anti-IL-12 mAb (50.5 ± 2.1% inhibition; $p < 0.01$; $n = 4$; Fig. 6*C*). Taken together, these data indicate that IFN-γ secretion in response to *T. gondii*-infected DC is largely dependent on the CD28 and CD40L pathways.

Next, we examined whether CD28 and CD40L signaling modulate IL-12-independent IFN-γ production triggered by DC plus viable *T. gondii*. Fig. 7 shows that after neutralization of IL-12 (incubation with anti-IL-12 mAb), addition of anti-CD40L mAb to T cells stimulated with md-DC and viable tachyzoites resulted in a moderate inhibition of IFN-γ production (30.9 ± 1.5% inhibition; $p < 0.01$; $n = 4$). Blockade of the CD80/CD86-CD28 interaction with CTLA-4-Ig induced marked inhibition of IFN-γ secretion (65.3 ± 3.9% inhibition; $p < 0.005$; $n = 4$). Simultaneous incubation with anti-CD40L mAb and CTLA-4-Ig further impaired IFN-γ production (80.1 ± 2.5; $p < 0.001$; $n = 4$). Thus, CD28 and, to a lesser extent, CD40L signaling control IL-12-independent IFN-γ secretion in response to *T. gondii*-infected DC.

*IL-12 production during T cell-*T. gondii*-infected DC interaction is dependent on CD40L*

In experiments parallel to those shown in Fig. 7, anti-CD40L mAb more prominently inhibited IFN-γ production if IL-12 was not neutralized (61.7 ± 1.3% inhibition; $n = 4$; see Fig. 6*A*). These results suggested that CD40-CD40L signaling during cognate interaction between T cells and *T. gondii*-infected DC may also influence IFN-γ secretion through regulation of IL-12 secretion. Therefore, we examined whether blockade of the CD40-CD40L pathway impairs IL-12 secretion triggered by *T. gondii*. Incubation of T cells with *T. gondii*-infected b-DC resulted in IL-12 p40 production (Fig. 8*A*). Addition of anti-CD40L mAb to these cells significantly inhibited IL-12 p40 secretion (70.4 ± 9.8% inhibition; $p < 0.03$; $n = 3$). To further confirm the role of CD40L signaling on IL-12 production, we studied the effects of anti-CD40L mAb on IL-12 p70 synthesis. Addition of anti-CD40L mAb to T cells incubated with md-DC infected with viable *T. gondii* remarkably inhibited IL-12 p70 secretion (86.6 ± 8.1% inhibition; $p < 0.01$; $n = 2$; Fig. 8*B*). Thus, T cell-dependent IL-12 production by *T. gondii*-infected DC is controlled by CD40-CD40L signaling.

Discussion

Inasmuch as DC appear to be the APC mainly responsible for generation of primary T cell responses (4), we considered it important to study how the interaction between DC and the intracellular protozoan *T. gondii* affects the generation of cell-mediated immunity against this pathogen. We have established that infection with viable tachyzoites, but not phagocytosis of killed parasites, induced activation of human DC. This process was accompanied by a strong T cell production of IFN-γ, a cytokine crucial for

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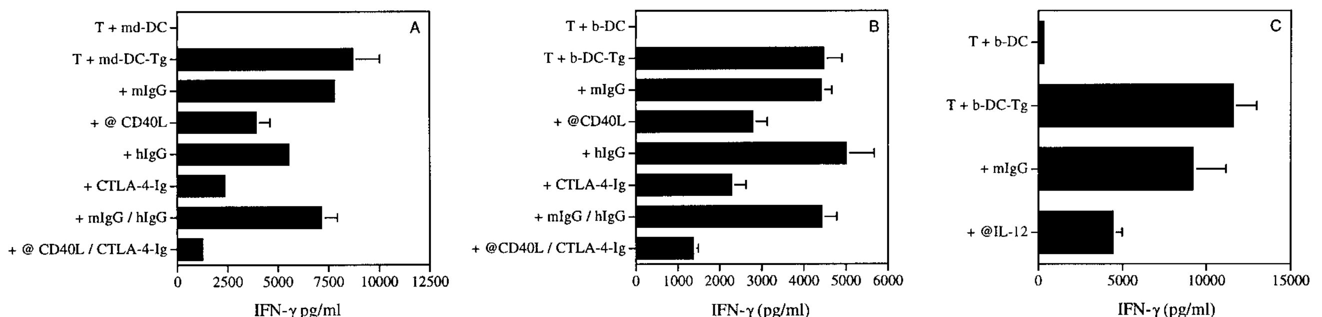

FIGURE 6. T cell production of IFN-γ in response to *T. gondii*-infected DC is dependent on CD40-CD40L and CD80/CD86-CD28 signaling. T cells (1×10^6/ml) were incubated with md-DC (*A*) or b-DC (*B* and *C*; both at 2.5×10^5/ml) that were either uninfected or infected with viable *T. gondii*. Anti-IL-12 mAb, anti-CD40L mAb, CTLA-4-Ig, or mouse or human IgG was added as indicated. The results of one representative experiment of four (*C*) or five (*A* and *B*) are shown.

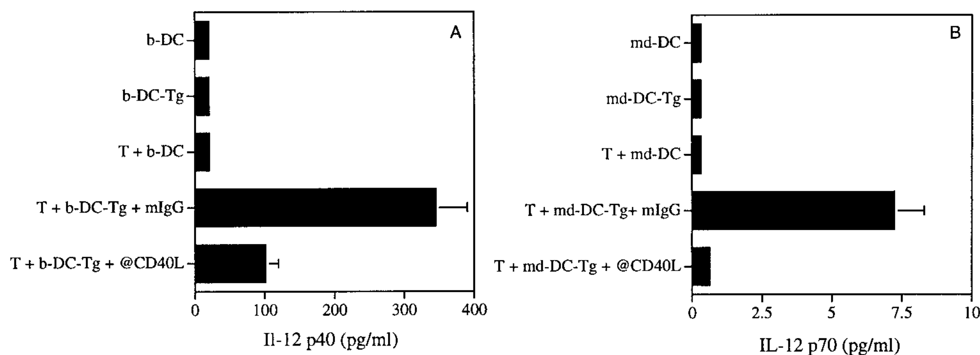

FIGURE 8. The CD40-CD40L pathway regulates IL-12 production during the interaction between T cells and *T. gondii*-infected DC. Uninfected or *T. gondii*-infected b-DC (*A*) or md-DC (*B*; all at 5×10^5/ml) were incubated either alone or in the presence of T cells (2×10^6/ml). Supernatants were collected after 24 h and were used to measure concentrations of either IL-12 p40 (*A*) or IL-12 p70 (*B*) by ELISA. Samples with undetectable IL-12 are shown as having a cytokine concentration half the lower limit of detection of the ELISA. Results of one representative experiment of two (*B*) or three (*A*) are shown.

protection against *T. gondii* (21, 22). Our studies indicate that T cell-*T. gondii*-infected DC cognate interaction is pivotal for IFN-γ production in response to the parasite. Secretion of this cytokine is regulated by CD40-CD40L-dependent IL-12 production. In addition, CD80/CD86-CD28 and CD40-CD40L interactions control IFN-γ synthesis through a mechanism that is operative even after neutralization of bioactive IL-12.

We report that incubation of immature DC with viable, but not killed, tachyzoites induced the appearance of CD115$^-$ CD40^{high} CD80^{high} CD86^{high} HLA-DR^{high} DC, a phenotype indicative of DC activation. Only 51% of activated md-DC contained intracellular tachyzoites, which were at different stages of degeneration. It remains to be determined whether human md-DC kill intracellular *T. gondii*. Such a finding would explain why not all activated dendritic cells contain intracellular parasites 18 h after infection. Of relevance to the *T. gondii*-induced md-DC activation is our demonstration that *T. gondii*-infected human monocytes also up-regulate the expression of CD40, CD80, CD86, and HLA-DR (2, 3). Certain microbes (Gram-positive and Gram-negative bacteria, mycobacteria, and measles virus) as well as LPS, CD40L, and cytokines such as TNF-α and IL-1β can induce DC maturation (7, 23–29). Although *T. gondii* up-regulates costimulatory ligands and HLA-DR expression on DC, the parasite may be unable to optimally induce the maturation marker CD83. However, full DC maturation (as defined by CD83 expression) may be achieved during T cell-DC interaction (30). Nevertheless, the capacity of human DC to up-regulate costimulatory ligands and MHC molecules after encountering viable, but not killed, preparations of *T. gondii* suggests that the parasite-DC interaction may influence the nature of the ensuing immune response.

IL-12 is a cytokine pivotal for control of *T. gondii* infection (31–33). We demonstrated that human DC secrete IL-12 p70 in response to viable, but not killed, *T. gondii* tachyzoites. However, IL-12 p70 was produced only after T cells were added to *T. gondii*-infected DC. Pertinent to these results are the reports that cognate interaction between human T cells and *T. gondii*-infected APC regulates IL-12 p40 secretion through CD40-CD40L signaling (3, 34). In contrast to studies in humans, experiments performed in mice indicate that in vitro production of IL-12 p40 by spleen cells can occur in the absence of T cells, and that i.v. administration of *T. gondii*-soluble Ags to mice results in a CD40L-independent transient in vivo production of IL-12 by DC (35). These contrasting results may be caused by host-related differences in the immune response to *T. gondii*.

We have demonstrated that IL-12 p70 production takes place after interaction between *T. gondii*-infected activated DC and T cells. Although it has been reported that only mature DC secrete IL-12 p70 (24), recent studies indicate that DC maturation does not necessarily imply that these cells will produce IL-12 (36). DC induced to mature in the presence of PGE$_2$ show impaired production of IL-12 p70 and promote the secretion of Th2-type cytokines by naive Th cells (36). Thus, it has been proposed that rather than maturation per se, the types of events that lead to DC maturation determine the polarizing capacity of DC (36). The nature of the signals triggered by *T. gondii* infection that cause DC activation remains to be characterized. Identification of the mechanisms through which *T. gondii* induces DC activation is likely to explain at least in part why this pathogen is a potent inducer of IL-12/IFN-γ secretion.

Although IL-12 is crucial for regulation of IFN-γ secretion, our studies indicate that the IFN-γ secretion by T cells stimulated with *T. gondii*-infected DC is in part independent of IL-12. These results are unlikely to be due to partial neutralization of IL-12. In parallel experiments, the anti-IL-12 mAb used in these studies neutralized the effect of 1 ng of rIL-12, a concentration far greater than the amount of IL-12 p70 secreted during T cell-*T. gondii*-infected DC interaction. Of relevance is the report that mice infected with the ts-4 strain of *T. gondii* secrete IFN-γ in an IL-12-independent manner (37). Moreover, this study suggested that class II-restricted T cells are involved in the IL-12-independent secretion of IFN-γ (37). It is interesting to point out that the in vitro reactivity to *T. gondii* of unprimed human $\alpha\beta$ T cells lies within the CD4$^+$ subset, and that this response requires MHC class II molecules (11). Thus, these similarities suggest an in vivo correlate to our results. IL-12-independent pathways for IFN-γ and Th1-type cytokine response have also been reported in murine models of viral infections (38, 39).

T. gondii mediates up-regulation of CD80, CD86, and CD40 on DC, a phenomenon that is likely to have important implications for the initiation of a T cell response against the pathogen. Indeed, blockade of the CD28 and CD40L pathways inhibits IFN-γ production in response to *T. gondii*-infected DC. Thus, our results suggest that these signaling pathways play an important role in the generation of protective immunity against the parasite in humans. Studies in CD28 and in CD40L knockout mice indicate that these animals are resistant to acute infection with the ME49 strain of *T. gondii* (40). It remains to be determined whether these results represent the development of compensatory mechanisms in the form of costimulation provided by alternative signaling pathways. Nevertheless, splenocytes from *T. gondii*-infected CD28 knockout mice exhibit impaired secretion of IFN-γ in response to *T. gondii*, and these animals show increased susceptibility to rechallenge

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with a virulent strain of *T. gondii* (40). In contrast to animal studies, data in humans reveal that patients with hyper-IgM syndrome, an immunodeficiency caused by lack of functional CD40L, exhibit impaired IL-12 and IFN-γ production in response to *T. gondii* (3). Moreover, these patients are at risk for the development of toxoplasmic encephalitis and disseminated toxoplasmosis (41–43). Therefore, the association between hyper-IgM syndrome and toxoplasmosis supports the idea that events that occur during the cognate interaction between T cells and APC (at least in the form of CD40-CD40L signaling) are important for the control of *T. gondii* infection in humans.

The demonstration that T cell production of IFN-γ in response to *T. gondii*-infected DC is regulated by both IL-12 and CD28 signaling is in agreement with the roles of these molecules in IFN-γ production in response to PHA (44) and the synergistic effects of IL-12 and CD28 stimulation on T cell cytokine secretion and proliferation (45, 46). Our studies revealed that CD28 costimulation also regulates IL-12-independent T cell secretion of IFN-γ in response to *T. gondii* (stimulation with DC plus viable *T. gondii* in the presence of anti-IL-12 mAb and stimulation with DC plus killed *T. gondii*). These results suggest that CD28 directly mediates T cell production of IFN-γ in response to *T. gondii*. Indeed, after neutralization of IL-12, a stimulatory anti-CD28 mAb increases IFN-γ secretion by PBMC incubated with PHA (44). Moreover, increased CD28-mediated costimulation can influence T cell cytokine production by preferentially promoting IFN-γ over IL-4 secretion (47).

CD40-CD40L interaction is not only involved in cytokine secretion by APC (48–50), but also modulates T cell function (51–54). Thus, the pleiotropic nature of this signaling pathway raised the possibility that CD40-CD40L interaction regulates IFN-γ secretion in response to *T. gondii*-infected DC through multiple mechanisms. Our results indicate that during the cross-talk between presumably unprimed human T cells and *T. gondii*-infected DC, CD40-CD40L signaling controls IFN-γ production through induction of IL-12 secretion. Of relevance is the demonstration that blockade of this pathway impairs IL-12 secretion when PBMC are incubated with *T. gondii* and when T cells from chronically infected individuals are stimulated with DC plus *T. gondii* (3, 34). However, our studies also suggest that CD40-CD40L interaction regulates IFN-γ secretion in response to DC plus *T. gondii* through an IL-12-independent mechanism. In this regard, CD40 can provide a costimulatory signal to T cells that results in increased IFN-γ and IL-2 production and enhanced T cell proliferation (51).

Collectively, our studies provide evidence of the importance of T cell-APC cognate interaction for the generation of IL-12/IFN-γ-dependent immunity against *T. gondii*. CD40-CD40L signaling triggers IL-12 secretion by *T. gondii*-infected DC, which, in turn, mediates T cell production of IFN-γ. In addition, CD80/CD86-CD28 and CD40-CD40L signaling controls IFN-γ production even in the absence of bioactive IL-12. These results suggest that CD80/CD86-CD28 and CD40-CD40L interactions also regulate IFN-γ production through direct T cell costimulation. It could be proposed that the reasons for the lower IFN-γ production after T cell stimulation with DC plus killed parasites are the absence of IL-12 p70 secretion as a result of the lack of activation of the CD40-CD40L pathway, and the lower levels of CD28- and CD40L-dependent costimulation (lower DC expression of CD40, CD80, and CD86).

Our results reveal that human DC discriminate between viable and nonviable preparations of *T. gondii*. The data indicate that the interaction between *T. gondii* and APC induces changes in infected APC, which, in turn, are pivotal for influencing cytokine response. This pathogen-APC-T cell interaction results in IL-12/IFN-γ production in situations where an IL-12/IFN-γ-dependent cell-mediated response would be appropriate (infection with viable tachyzoites), whereas no such response is triggered when encountering nonviable parasites. These data are reminiscent of the "danger" model, where distinction between noxious and harmless stimuli is made by APC through up-regulation of costimulatory ligands (55). Our results suggest that the immune system is capable of distinguishing between viable and killed *T. gondii* tachyzoites through modulation of CD28 and CD40L signaling as a result of the interaction between viable *T. gondii* and DC.

A recent report indicates that mice immunized with DC pulsed with TLA were protected against challenge with *T. gondii* tissue cysts (56). Resistance to infection was accompanied by ex vivo IFN-γ secretion in response to TLA (56). However, these studies were performed using splenic DC after overnight incubation in complete medium. Since such culture conditions result in DC maturation, data from these animals studies do not conflict with our results. The fact that infection with viable *T. gondii* bradyzoites, rather than immunization with TLA, is necessary for acquisition of resistance to tachyzoites of a virulent strain of the parasite (57) suggests that our results may be relevant to the in vivo immune response to the parasite.

Since our studies were performed using presumably unprimed T cells (*T. gondii*-seronegative donors), the results indicate that human DC are likely to be important for polarization of the T cell response against *T. gondii*. Indeed, DC appear to be the initial source of IL-12 in mice exposed in vivo to *T. gondii* Ags (35). Our data support the hypothesis that signals provided during the interaction between microbial organisms and DC influence the generation of a primary immune response. In the case of *T. gondii*, these events may explain why this pathogen is a potent inducer of IL-12/IFN-γ secretion. In contrast, by inhibiting DC maturation and impairing the capacity of these cells to process Ag (58–60), other pathogens may have evolved mechanisms of defense based on inhibition of recognition by the immune system. Further studies of the pathogen-APC-T cell interaction may unravel mechanisms that determine the nature of the immune response elicited.

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