CD40-CD40 Ligand Interaction Is Central to Cell-Mediated Immunity Against *Toxoplasma gondii*: Patients with Hyper IgM Syndrome Have a Defective Type 1 Immune Response That Can Be Restored by Soluble CD40 Ligand Trimer

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CD40-CD40 Ligand Interaction Is Central to Cell-Mediated Immunity Against Toxoplasma gondii: Patients with Hyper IgM Syndrome Have a Defective Type 1 Immune Response That Can Be Restored by Soluble CD40 Ligand Trimer

Carlos S. Subauste,* Matthew Wessendorp,* Ricardo U. Sorensen,† and Lily E. Leiva†

Cell-mediated immunity that results in IL-12/IFN-γ production is essential to control infections by intracellular organisms. Studies in animal models revealed contrasting results in regard to the importance of CD40-CD40 ligand (CD40L) signaling for induction of a type 1 cytokine response against these pathogens. We demonstrate that CD40-CD40L interaction in humans is critical for generation of the IL-12/IFN-γ immune response against Toxoplasma gondii. Infection of monocytes with T. gondii resulted in up-regulation of CD40. CD40-CD40L signaling was required for optimal T cell production of IFN-γ in response to T. gondii. Moreover, patients with hyper IgM (HIGM) syndrome exhibited a defect in IFN-γ secretion in response to the parasite and evidence compatible with impaired in vivo T cell priming after T. gondii infection. Not only was IL-12 production in response to T. gondii dependent on CD40-CD40L signaling, but also, patients with HIGM syndrome exhibited deficient in vitro secretion of this cytokine in response to the parasite. Finally, in vitro incubation with agonistic soluble CD40L trimer enhanced T. gondii-triggered production of IFN-γ and, through induction of IL-12 secretion, corrected the defect in IFN-γ production observed in HIGM patients. Our results are likely to explain the susceptibility of patients with HIGM syndrome to infections by opportunistic pathogens. The Journal of Immunology, 1999, 162: 6690–6700.

Increasing evidence points toward the importance of cognate interaction between T cells and APC during cell-mediated immunity. CD40 is a molecule pivotal to this cell-to-cell communication. This surface glycoprotein is expressed on professional APC as well as on nonhematopoietic cells (1–4). In contrast, its counterreceptor, CD40 ligand (CD40L),3 is expressed primarily on activated CD4+ T cells (5, 6). The importance of CD40-CD40L interaction for the biology of B cells (proliferation of B cells, Ig synthesis, isotype switch, and germinal center formation) is well established (7). More recent data indicate that these molecules are also central to T cell-mediated activation of monocyte/macrophages and dendritic cells. In this regard, ligation of CD40 on these cells stimulates cytokine secretion, increases expression of adhesion molecules, induces monocyte tumoricidal activity, and protects them from apoptosis (2, 8, 9). Moreover, studies of patients with hyper IgM (HIGM) syndrome, an immunodeficiency caused by defective CD40L expression, indicate that these patients not only exhibit impaired humoral immunity but also suffer from infections associated with defects in T cell-mediated immunity (10). In addition, impaired production of IFN-γ and IL-12 and increased mortality have been demonstrated in murine models of leishmanial infection in CD40 and CD40L knockout mice (11–13). Together, these observations suggested that CD40-CD40L interaction is required for induction of IL-12/IFN-γ-mediated protective immunity against intracellular pathogens. However, recent reports indicate that this type of immune response can be generated in CD40L knockout mice infected with Mycobacterium tuberculosis and Histoplasma capsulatum (14, 15). Thus, the extent to which CD40-CD40L is involved in generation of protective IL-12/IFN-γ-dependent cell-mediated immunity against intracellular pathogens is unclear.

Toxoplasma gondii is an obligate intracellular protozoan that commonly infects humans throughout the world. Cell-mediated immunity with resulting IFN-γ production is central to control of T. gondii infection (16, 17). Indeed, it appears that such an immune response controls parasite multiplication during the acute phase of infection and promotes establishment of the chronic (quiescent) phase of infection. Our previous demonstration that unprimed human T cells proliferate and secrete IFN-γ in vitro when incubated with T. gondii-infected monocytes (18, 19) indicates that this in vitro model is appropriate for the study of factors responsible for production of IFN-γ by T cells.

We have recently demonstrated that signaling through the CD28–CD80/CD86 system as a result of cognate interaction between T. gondii-infected human monocytes and T cells from naive individuals is critical for IFN-γ production (18). In an attempt to better understand the cross-talk between T cells and APC infected with intracellular pathogens, we have investigated the role of CD40-CD40L interaction during the immune response to T. gondii. We demonstrate that through induction of IL-12 secretion, CD40-CD40L signaling is critical for the generation of an IFN-γ
response against *T. gondii* in humans. To our knowledge, this study demonstrates for the first time that patients with HIGM syndrome have defective IFN-γ and IL-12 secretion in response to an intracellular organism, which can be restored in vitro by agonistic soluble CD40L trimer (CD40LT). Impaired production of these cytokines may explain susceptibility of patients with HIGM syndrome to infections caused by opportunistic pathogens.

**Materials and Methods**

**mAbs and cytokines**

The following mAbs were used for cell purifications: anti-CD2, anti-CD3, anti-CD8, anti-CD14, anti-CD56 (all from Becton Dickinson, San Jose, CA), anti-CD16 (Medarex, Annandale, NJ), anti-CD19 (Coulter, Hialeah, FL), anti-CD66b (Immunotech, Westbrook, MA), and anti-glycophorin A (10F7 MN; a gift from Rene de Waal Malefyt, DNAX Research Institute, Palo Alto, CA). Anti-CD14 (RM052, Immunotech), anti-CD40 (M2; gift from Immunex, Seattle, WA), anti-CD40L (M90; gift from Immunex), CTLA-4-Ig (gift from Bristol-Myers Squibb, Princeton, NJ) (20), anti-IFN-γ (R&D Systems, Minneapolis, MN), and anti-IL-12 (C8.6, gift from Giorgio Trinchieri, The Wistar Institute, Philadelphia, PA) were used in functional assays (all at 10 μg/ml unless stated otherwise). Isotype-matched mAbs and control IgG1 were obtained from Pharmingen (San Diego, CA) and Sigma (St. Louis, MO), respectively. Human CD40LT was a gift from Immunex. IFN-γ was obtained from R&D Systems. IL-12 was a gift from Genetics Institute (Cambridge, MA).

The following conjugated Abs were used for flow cytometry (purchased from Becton Dickinson, except when indicated): FITC-anti-CD3, FITC-anti-CD40 (PharMingen), FITC-anti-HLA-DR, PE-anti-CD16, PE-anti-CD56, PE-anti-CD80 (PharMingen) or PE-HLA-DR (Caltag, South San Francisco, CA); and cydan 5 (Cy5)-PE-anti-CD14 (Se-rotec, Oxford, U.K.).

**Cell purifications**

Using centrifugation on Ficoll-Hypaque gradients (Pharmacia LKB Biotechnology, Piscataway, NJ), PBMC were isolated fromuffy coats of heparinized blood of healthy volunteer 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 (21). Except when noted, samples used had no demonstrable *T. gondii* IgG or IgM Abs. In some experiments, blood from chronically infected but otherwise healthy adults was used (positive anti-*T. gondii* IgG and negative anti-*T. gondii* IgM). In addition, PBMC were obtained from three patients followed at Children’s Hospital (New Orleans, LA) with a diagnosis of HIGM syndrome, one of whom was chronically infected with *T. gondii* and had prior history of toxoplasmic encephalitis (RG) (22). Diagnosis of HIGM syndrome was made based on established criteria, which included lack of CD40L expression on activated T cells (10).

To obtain purified monocytes, PBMC were incubated with the following mAb: anti-CD2, anti-CD3, anti-CD8, anti-CD19, anti-CD56, anti-CD66b, and anti-glycophorin A. After addition of magnetic beads coated with anti-mouse IgG (Dynal, Great Neck, NY), rosetting cells were removed with a magnet (Dynab) (18). Populations obtained were ≥92% CD14+ monocytes. Monocytes were purified further by incubation with Cy5-PE-conjugated anti-CD14 mAb followed by FACS sorting. This procedure resulted in populations of highly purified CD14+ monocytes (≥99% by flow cytometry).

Resting T cells (≤99% CD3+) were obtained from nylon-wool nonadherent PBL that were incubated with anti-CD16 plus anti-CD56 and subjected to depletion using immunomagnetic beads (18). Cells were cultured in teflon vessels using complete medium (CM) consisting of RPMI 1640 supplemented with 10% dye-test negative human AB serum (Gemini Biological Products, Calabasas, CA). The following conjugated Abs were used for flow cytometry: anti-CD2, anti-CD3, anti-CD8, anti-CD14, anti-CD56, anti-CD66b, and anti-glycophorin A. After addition of magnetic beads coated with anti-mouse IgG (Dynal, Great Neck, NY), rosetting cells were removed with a magnet (Dynab) (18). Populations obtained were ≥92% CD14+ monocytes. Monocytes were purified further by incubation with Cy5-PE-conjugated anti-CD14 mAb followed by FACS sorting. This procedure resulted in populations of highly purified CD14+ monocytes (≥99% by flow cytometry).

**Cytokine assays**

Purified peripheral blood T cells (1 × 10^6/ml) were incubated in 96-well plates with different concentrations of either uninfected or *T. gondii*-infected monocytes or PBMC. Abs were added to monocytes 30 min before incubation with T cells. Unless indicated, these reagents were used at 10 μg/ml. Supernatants were collected at 72 h and stored at −70°C. Concentrations of IFN-γ were measured by ELISA (Endogen, Cambridge, MA). The lower limit of detection for this ELISA was 39 pg/ml. Data is presented as mean of triplicate wells ± SEM. IFN-γ was not detected in supernatants obtained from wells that lacked T cells and contained only monocytes with or without *T. gondii* antigenic preparations. Compared with production of IFN-γ in the presence of T cells, γ-irradiated *T. gondii*-infected PBMC incubated alone always secreted <20% the amount of IFN-γ present when T cells were added to the wells. IFN-γ production was also studied after stimulation of PBMC (1 × 10^6/ml) with PMA (10 ng/ml; Calbiochem, La Jolla, CA) plus ionomycin (1 μg/ml; Sigma).

For assays of IL-12 production, PBMC (2 × 10^6/ml) were incubated with varying concentrations of tachyzoites or with *Staphylococcus aureus* Cowan I strain (0.0075%, Pansorbin; Calbiochem) plus IFN-γ (100 U/ml). Supernatants were collected after 24 h and stored at −70°C. Concentrations of IL-12 p40 were measured by ELISA (R&D Systems). The lower limit of detection for this ELISA was 39 pg/ml.

**Results**

**T cell proliferation assays**

T cells (5 × 10^5/ml) were incubated for 7 days in 96-well plates with varying concentrations of either infected or uninfected PBMC, or with PBMC plus varying concentrations of TLA, as described in Results (19). After labeling with ^[3]H^]thymidine during the final 18 h, samples were harvested, and radioactivity was measured in a β-scintillation counter (23). Results are expressed as mean cpm of ^[3]H^]thymidine incorporation of triplicate wells ± SEM.

**Statistical analysis**

Statistical significance was assessed by Student’s t test.

**Experiments were conducted to determine whether *T. gondii* affects expression of CD40 on human monocytes. These studies were performed using monocytes obtained by negative depletion (≥92% CD14+), as well as the highly purified monocyte populations (>99% CD14+). Incubation with tachyzoites resulted in the appearance of two subpopulations of monocytes: one that persisted with low expression of CD40 (CD40<low>) and another with increased expression of CD40 (CD40<high>) (Fig. 1A). Up-regulation of CD40 was accompanied by a progressive decrease in expression of CD14. On average, CD40<high> monocytes had 3.8 times higher expression of CD40 than CD40<low> monocytes (MFI, 32.7 ± 3.1 vs 8.6 ± 1.1; n = 10). Up-regulation of expression of CD40 was not a nonspecific effect caused by phagocytosis of foreign particles since incubation with latex beads failed to alter expression of CD40 (data not shown). To determine whether incubation with viable parasites is required for up-regulation of CD40, monocytes were cultured with either viable or killed tachyzoites, or with TLA. As shown in Fig. 1B, only incubation with viable tachyzoites resulted in induction of CD40<high> monocytes. Moreover, the cMFI for CD40 on these cells was at least 3.5 times higher than that on monocytes incubated with either killed tachyzoites, TLA, or CM alone (Fig. 1C).

We have recently reported that monocytes infected with *T. gondii* tachyzoites become CD80+ and display increased expression of IFN-γ and IL-12.
of CD86 (CD86\textsuperscript{high}) (18). To determine whether CD40\textsuperscript{high} monocytes are also CD80\textsuperscript{low} and/or CD86\textsuperscript{high}, monocytes incubated with live tachyzoites were stained with Cy5-PE-anti-CD14 plus FITC-anti-CD40 and either PE-anti-CD80 or PE-anti-CD86 mAbs. As shown in Table I, both CD80\textsuperscript{low} and CD86\textsuperscript{high} monocytes displayed increased levels of expression of CD40 (n = 3). Because we previously demonstrated that HLA-DR molecules are required for the in vitro response against T. gondii (Tg), paraformaldehyde-killed T. gondii (PFKTg), TLA, IFN-γ (100 U/ml), or CM alone (Control). The percentage of CD40\textsuperscript{high} monocytes and the cMFI were determined by flow cytometry. A representative experiment of 4–10 is shown.

Since we have previously demonstrated that infection of monocytes with T. gondii tachyzoites is responsible for induction of CD80 and up-regulation of CD86, these results suggest that infection with T. gondii also causes up-regulation of CD40. Experiments using Transwell inserts indicated that soluble factors alone are unlikely to mediate CD40 up-regulation. Incubation of monocytes with T. gondii tachyzoites separated from monocytes alone by a membrane with 0.4-μm pores resulted in up-regulation of CD40 only in monocytes in direct contact with the parasite (data not shown). The up-regulation of CD40 was not attributable to LPS, since preparations of T. gondii did not contain detectable levels of LPS (<0.015 EU/ml by amebocyte Limulus assays), and addition of a mAb against CD14 (LPS receptor), which neutralizes the effects of LPS on human monocytes (24), did not inhibit the effects of T. gondii on the levels of expression of CD40 (data not shown).

To determine the kinetics of T. gondii-mediated up-regulation of CD40, monocytes incubated with viable tachyzoites or CM alone were analyzed by flow cytometry at different time points after onset of in vitro culture. As shown in Fig. 2A, induction of CD40\textsuperscript{high} monocytes became apparent 12 h after incubation with T. gondii and reached a peak at 24 h. Similarly, analysis of levels of expression of CD40 indicated that maximal T. gondii-mediated up-regulation of this molecule was observed at 24 h (Fig. 2B). Thus, these data indicate that T. gondii triggers a rapid up-regulation of expression of CD40 on monocytes.

### Table I. T. gondii-induced CD40\textsuperscript{high} monocytes are CD80\textsuperscript{low}, CD86\textsuperscript{high} and HLA-DR\textsuperscript{high}\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>CD40\textsuperscript{high}</th>
<th>CD40\textsuperscript{low}</th>
<th>Fold Increase (cMFI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD80</td>
<td>197.0 ± 22.2</td>
<td>3.2 ± 0.2</td>
<td>60.4 ± 3.9</td>
</tr>
<tr>
<td>CD86</td>
<td>2346.0 ± 224.6</td>
<td>304.3 ± 36.6</td>
<td>7.73 ± 0.2</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>1223.0 ± 235.0</td>
<td>294.0 ± 13.0</td>
<td>4.2 ± 0.7</td>
</tr>
</tbody>
</table>

\textsuperscript{a} After 48 hr of in vitro culture, monocytes were subjected to three-color FACS analysis using Cy5-PE-anti-CD14 plus FITC-anti-CD40 and either anti-CD80 or anti-CD86 or anti-HLA-DR mAbs. Results of one representative experiment of three are shown.
addition of either anti-CD40 or anti-CD40L mAbs to T cells stimulated with an optimal concentration of T. gondii-infected monocytes resulted in a moderate, but significant inhibition of IFN-γ production (38 ± 7%; range from 23 to 53%) (p < 0.04; n = 5). It appeared unlikely that the partial inhibition observed was due to an inadequate dose of mAbs since, at the same concentration, anti-CD40L mAb neutralized (100% inhibition) the effect of CD40LT (500 ng/ml) on production of IFN-γ in response to T. gondii (see below). Next, we examined whether the extent of inhibition of IFN-γ secretion could be affected by the dose of infected monocytes used to stimulate T cells. As the concentration of infected monocytes was decreased, we observed a progressive increase in the inhibitory effects of anti-CD40 and anti-CD40L mAbs leading to a markedly impaired IFN-γ production (80 ± 9% inhibition) (p < 0.02; n = 3) (Fig. 3B). Taken together, our data indicate that CD40-CD40L signaling is required for optimal T cell stimulation. In parallel experiments, we studied the effects of anti-CD40 and anti-CD40L mAbs on T cell proliferation triggered by T. gondii-infected monocytes. Whereas these mAbs induced a consistent inhibition in IFN-γ production, inhibition in T cell proliferation (from 31 to 65% inhibition) was observed in only two of six experiments, even when low concentrations of infected monocytes were used (data not shown).

We have previously demonstrated that the CD28-CD80/CD86 system provides costimulation that is critical for the T cell response against T. gondii (18). Therefore, we studied whether this signaling pathway acts in concert with the CD40-CD40L system during the immune response to T. gondii. Fig. 4 shows that adding increasing concentrations of CTLA4Ig to T cells stimulated with T. gondii-infected monocytes results in progressive inhibition of T cell secretion of IFN-γ. When comparing the combination of CTLA4Ig plus anti-CD40 mAb to CTLA4Ig plus isotype control mAb, addition of anti-CD40 mAb to CTLA4Ig resulted in significant further inhibition of IFN-γ production (p < 0.03; n = 3). Thus, CD40-CD40L and CD28-CD80/CD86 pathways cooperate in the modulation of the T cell response against T. gondii-infected monocytes. T cells from a patient with HIGM syndrome chronically infected with T. gondii react to the parasite in vitro in a manner similar to that of T cells from T. gondii-seronegative individuals.

To determine whether the absence in vivo of CD40-CD40L signaling affects T cell responses to T. gondii, we studied the parasite-triggered T cell proliferation and IFN-γ secretion by T cells isolated from an individual with HIGM syndrome and chronic T. gondii infection (RG) (22). These results were compared with those obtained after stimulation of T cells from healthy subjects. We have previously demonstrated that resting αβ T cells from both individuals chronically infected with T. gondii and seronegative individuals proliferate in vitro when stimulated with either PBMC infected with T. gondii or pulsed with TLA (19). However, only αβ T cells from chronically infected individuals exhibit in...
vitro reactivity to low concentrations of T. gondii antigenic preparations (19). As shown in Fig. 5, A–D, similar results were obtained when resting, unseparated T cells from chronically infected and seronegative individuals were stimulated with decreasing concentrations of T. gondii-infected PBMC or PBMC plus decreasing concentrations of TLA. In parallel experiments, T cells from a patient with HIGM syndrome chronically infected with T. gondii exhibited proliferation only to high doses of T. gondii Ag preparations (Fig. 5, E–F). This proliferative response diminished remarkably as the concentrations of infected PBMC or TLA were decreased.

Study of IFN-γ production by T cells in response to T. gondii showed a pattern of response similar to that observed with T cell proliferation. Whereas T cells from both uninfected and chronically infected healthy subjects secreted IFN-γ after stimulation with T. gondii-infected PBMC, only T cells from chronically infected individuals produced IFN-γ after stimulation with low concentrations of infected PBMC (Fig. 6). T cells from chronically infected individuals secreted high amounts of IFN-γ even after stimulation with low concentrations of TLA. In contrast, either low

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**FIGURE 4.** CD40-CD40L and CD28-CD80/CD86 pathways cooperate for T cell production of IFN-γ. T cells were incubated with T. gondii-infected autologous monocytes. Cells were cultured with increasing concentrations of CTLA4Ig in the presence of 10 μg/ml of either anti-CD40 or mouse IgG1 control mAbs. Human IgG did not significantly affect IFN-γ secretion. Results of one representative experiment of three are shown.

**FIGURE 5.** T cells from a T. gondii-chronically infected patient with HIGM syndrome have in vitro reactivity against T. gondii that resembles that of T cells from T. gondii-seronegative individuals. T cells from healthy controls, either T. gondii-seronegative (A and B) or T. gondii-seropositive (C and D), or from a patient with HIGM syndrome (RG) chronically infected with T. gondii (E and F) were incubated with either decreasing concentrations of T. gondii-infected PBMC or uninfected PBMC plus decreasing concentrations of TLA. Results are representative of those obtained with six seronegative donors, four seropositive donors, and three independent experiments performed with cells from the patient with HIGM syndrome.
or undetectable amounts of IFN-γ were measured when T cells from seronegative individuals were stimulated with a high concentration of TLA. In parallel experiments, T cells from the patient with HIGM syndrome produced IFN-γ only after incubation with high concentrations of T. gondii-infected PBMC and secreted low amounts of this cytokine after stimulation with PBMC plus a high concentration of TLA. Taken together, these results indicate that, despite prior exposure to T. gondii in vivo, T cells from the patient with HIGM syndrome react in a manner similar to T cells isolated from seronegative individuals.

**Patients with HIGM syndrome have defective in vitro production of IFN-γ in response to T. gondii, which is restored by CD40LT**

Our studies of IFN-γ production described above also indicated that T cells from the patient with HIGM syndrome had a defect in IFN-γ secretion in response to T. gondii-infected cells. Inasmuch as T cells from this patient reacted in a manner similar to those from seronegative donors, this group of controls was used for comparative purposes. IFN-γ production by the patient’s T cells in response to an optimal concentration of infected PBMC was always <24% of that by T cells from any of the six healthy unexposed controls tested (average concentrations: 2, 562 ± 183 vs 14, 118 ± 1, 302, respectively) (p < 0.0005). To confirm that patients with HIGM syndrome suffer from a defect in IFN-γ production in response to T. gondii, PBMC from three patients with this immunodeficiency were incubated with increasing concentrations of tachyzoites. Compared with healthy controls, all of these patients exhibited a markedly reduced in vitro production of IFN-γ in response to T. gondii (p < 0.0001) (Fig. 7A). This defect was not caused by an intrinsic inability to secrete this cytokine, since PBMC incubated with PMA plus ionomycin produced remarkable amounts of IFN-γ (HIGM: 16, 995 ± 1, 918 pg/ml; Controls: 17, 277 ± 1, 495) (p > 0.9).

To further determine the functional relevance of CD40-CD40L signaling during the immune response against T. gondii, we studied the effects of CD40LT on T cell production of IFN-γ in response to T. gondii-infected cells. A dose-dependent enhancement in IFN-γ production was observed when T cells from seronegative donors were stimulated with T. gondii-infected PBMC in the presence of CD40LT (Fig. 7B). The stimulatory effect of CD40LT was caused by signaling through the CD40-CD40L pathway, since coincubation with anti-CD40L mAb resulted in 85 ± 4% inhibition of the effect of CD40LT (data not shown). Next, we determined
whether CD40LT can correct the defective in vitro IFN-γ secretion observed in patients with HIGM syndrome. After addition of CD40LT, production of IFN-γ by PBMC from patients with HIGM syndrome incubated with T. gondii was no longer significantly different from that by cells from healthy seronegative controls (p > 0.05) (Fig. 7C). Similarly, after stimulation with an optimal concentration of T. gondii-infected PBMC plus CD40LT, T cells from one of these patients (RG) produced amounts of IFN-γ that approached those secreted by T cells from six healthy seronegative controls (10,814 ± 2,651 vs 19,326 ± 3,902, respectively) (p > 0.05).

Optimal production of IL-12 in response to T. gondii requires CD40-CD40L signaling

We conducted a series of experiments aimed at determining if defective IFN-γ production observed in patients with HIGM syndrome was caused by impaired IL-12 secretion. As an initial step, we ascertained whether T. gondii triggers IL-12 production and whether this response is dependent on CD40-CD40L signaling. Fig. 8A shows that there was IL-12 secretion when PBMC from healthy seronegative donors were incubated with T. gondii. Moreover, addition of anti-CD40L mAb to PBMC stimulated with an optimal concentration of tachyzoites resulted in a significant inhibition in IL-12 production (56.3 ± 4.4% inhibition) (p < 0.02; n = 4) (Fig. 8B). Next, we determined whether this inhibitory effect was caused through the effects of anti-CD40L mAb on IFN-γ secretion, rather than through a direct effect on IL-12 production. Although supernatants collected 24 h after incubation of PBMC with the parasite contained IFN-γ (2.2 ± 0.7 ng/ml), stimulation with T. gondii in the presence of anti-IFN-γ mAb failed to affect IL-12 production (Fig. 8B). Thus, the inhibitory effect of anti-CD40L mAb on IL-12 production was not mediated through decreased IFN-γ secretion. At the concentration used, anti-IFN-γ mAb abrogated (97 ± 2% inhibition) the stimulatory effect of 50 ng/ml of IFN-γ on LPS-mediated IL-12 production by PBMC (data not shown).

The partial inhibition of IL-12 production caused by anti-CD40L was unlikely to be due to an inadequate dose of mAb since, at the same concentration, anti-CD40L mAb neutralized (99 ± 1.2% inhibition) the effect of CD40LT (1 μg/ml) on IL-12 secretion in response to T. gondii. Thus, we examined whether the dose of tachyzoites used to stimulate IL-12 production could affect the extent of inhibition of secretion of this cytokine mediated by anti-CD40L mAb. As the concentration of tachyzoites was decreased, we observed a progressive increase in the inhibitory effect of anti-CD40L mAb leading to markedly impaired IL-12 secretion (89 ± 8% inhibition) (p < 0.02; n = 4) (Fig. 8C). Together, our data
and 89 6 enhanced production of IFN-g CD40LT were significantly inhibited by anti-IL-12 mAb (82 6 6 repeated at least twice. Results shown in T. gondiiduction of IFN-
We determined whether the increase in IFN-
further address the role of IL-12 in the regulation of IFN-
mainly through increased secretion of bioactive IL-12. Finally, to
IFN-
thesis, we determined whether rIL-12 could correct the defective
production seen in PBMC from patients with HIGM syndrome, which approached that by PBMC from seronegative healthy controls.

Discussion

The results of this study demonstrate that CD40-CD40L interaction plays an important role in the generation of an IL-12/IFN-γ immune response against T. gondii. Production of these cytokines was inhibited by blockade of CD40-CD40L signaling. Moreover, patients with HIGM syndrome exhibited a deficiency in the in vitro secretion of these cytokines in response to the parasite that was restored by addition of exogenous CD40L. The regulatory activity of CD40L on IFN-γ production in response to T. gondii was mediated primarily by CD40L-dependent release of IL-12. In addition, our studies of in vitro reactivity of T cells from a patient with HIGM syndrome who was chronically infected with T. gondii strongly suggest that another crucial role of the CD40-CD40L pathway may be that of mediating T cell priming during the in vivo immune response against T. gondii.

We have previously demonstrated that human monocytes have the capacity to discriminate between viable and dead preparations

CD40LT enhances IFN-γ production through IL-12 secretion

We determined whether the increase in IFN-γ synthesis caused by CD40LT was mediated through IL-12 production. PBMC from healthy seronegative individuals were stimulated with T. gondii with or without CD40LT in the presence of a neutralizing anti-IL-12 mAb. Fig. 10 reveals that both the T. gondii-mediated production of IFN-γ and the increased secretion of IFN-γ caused by CD40LT were significantly inhibited by anti-IL-12 mAb (82 ± 9% and 89 ± 3% inhibition, respectively) (p < 0.01; n = 3). Thus, the enhanced production of IFN-γ caused by CD40LT was mediated mainly through increased secretion of bioactive IL-12. Finally, to further address the role of IL-12 in the regulation of IFN-γ synthesis, we determined whether rIL-12 could correct the defective IFN-γ production seen in PBMC from patients with HIGM syn-

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of *T. gondii*. Thus, human monocytes acquire expression of CD80 and up-regulate expression of CD86 in response to infection with viable tachyzoites but not in response to phagocytosis of killed parasites or incubation with TLA (18). We now report that, after infection with viable *T. gondii* tachyzoites, human monocytes also up-regulate expression of CD40. The fact that infected monocytes are CD80<sup>+</sup>, CD86<sup>high</sup>, CD40<sup>high</sup>, and HLA-DR<sup>high</sup> indicates that infection with *T. gondii* activates human monocytes. This effect on monocytes may explain, at least in part, the proliferation and production of IFN-γ by unprimed T cells in response to highly purified monocytes (>99% CD14<sup>+</sup>) infected with *T. gondii* (18). It has been reported that *T. gondii* affects expression of surface molecules on monocytes through IFN-γ secretion (25). However, the up-regulation of CD40 that we observed is likely caused primarily by infection with the parasite rather than by secretion of soluble factors. Moreover, a neutralizing Ab against IFN-γ failed to affect modulation of monocyte expression of CD40 mediated by *T. gondii* (C.S.S., unpublished observations).

Blockade of CD40-CD40L signaling inhibited T cell production of IFN-γ in response to monocytes infected with *T. gondii*. This inhibitory effect was particularly striking at concentrations of infected APC that led to submaximal T cell response, conditions that may more closely mimic the early stages of the in vivo immune response against the parasite. The fact that the CD40-CD40L system can affect the function of both CD4<sup>+</sup> T cells and APC raised the possibility that the mechanism(s) through which this pathway regulates IFN-γ production in response to *T. gondii* may be multifactorial. Thus, CD40L induces not only IL-12 production during cognate interaction between T cells and APC (26–28), but can also act as a direct T cell stimulant (29). Our studies of the modulation of intracellular production of IFN-γ in response to *T. gondii* in humans demonstrate that IFN-γ production is largely dependent on IL-12, in a manner similar to the murine immune response against the parasite (30–32); the CD40-CD40L pathway is required for optimal IL-12 secretion; and neutralization of IL-12 almost completely ablates the stimulatory effect of CD40LT on IFN-γ production. Therefore, our results indicate that CD40L modulates IFN-γ production primarily through its effect on IL-12 secretion rather than through direct T cell stimulation. However, although CD40-CD40L signaling is important for optimal IL-12 production in response to *T. gondii*, blockade of this pathway failed to abrogate IL-12 secretion when PBMC were incubated with a high concentration of tachyzoites, and PBMC from patients with HIGM syndrome secreted low amounts of this cytokine. Thus, it appears that there are other factors that regulate IL-12 production in response to *T. gondii*. In this regard, i.v. administration of a preparation of *T. gondii* soluble Ags resulted in a transient, CD40-CD40L-independent production of IL-12 by murine dendritic cells (33). In addition, bacterial products, such as LPS and heat-killed *Listeria monocytogenes*, induce IL-12 secretion without signaling through CD40 (34). The existence of CD40L-independent mechanism(s) of IL-12 production during infections with certain intracellular pathogens may explain the generation of a type 1 immune response in CD40L knockout mice infected with *M. tuberculosis* and *H. capsulatum* (14, 15).

We have previously demonstrated that CD28-CD80/CD86 signaling is critical for generation of a T cell response against *T. gondii* (18). We now report that this pathway acts in concert with the CD40-CD40L system for the induction of optimal T cell production of IFN-γ in response to *T. gondii*-infected monocytes. Pertinent to our studies is the demonstration that blockade of both CD40L and CD28 pathways is required for effective inhibition of T cell responses (35–37). It has been proposed that CD40L regulates T cell activation through induction/up-regulation of CD80/CD86 on APC, which in turn provide costimulation to T cells through CD28 (38, 39). The evidence that simultaneous blockade of the CD28 and CD40L pathways results in further inhibition of IFN-γ production suggests that the mechanism(s) by which these two signaling systems modulate IFN-γ production during *T. gondii* infection are, at least in part, independently regulated.

Further evidence of the importance of CD40-CD40L signaling comes from our studies with patients with HIGM syndrome. The recent reports of patients with HIGM syndrome who developed toxoplasmic encephalitis (10, 22, 40) (a disease seen only in patients with depressed cell-mediated immunity) strongly suggests that the CD40-CD40L system is crucial for development of protective immunity against the parasite in humans. Indeed, we demonstrate that patients with HIGM syndrome have a defect in production of IL-12 and IFN-γ in response to *T. gondii*. Pertinent to our results is the demonstration that CD40-CD40L signaling is required for differentiation of Th1 cells but not Th2 cells (41). It is interesting to note that, although decreased, we did detect *T. gondii*-mediated IFN-γ production by T cells and PBMC from patients with HIGM syndrome. We have recently demonstrated that induction/up-regulation of CD80 and CD86 on *T. gondii*-infected monocytes is associated with T cell production of IFN-γ (18). Indeed, monocytes from patients with HIGM syndrome up-regulate co-stimulatory ligands in response to *T. gondii* (C.S.S., unpublished observations). Thus, CD28-CD80/CD86 signaling may explain secretion of IFN-γ in the absence of CD40L. In this regard, administration of CD80-bearing APC restores IFN-γ production in CD40L knockout mice (38), and direct CD28 stimulation induces IFN-γ secretion in the absence of CD40-CD40L signaling (42).

We demonstrate that T cells from a patient with HIGM syndrome and chronic *T. gondii* infection proliferate and produce IFN-γ in response to the parasite in a manner similar to that of T cells from healthy seronegative individuals. These results were not
due to immunosuppression associated with acute *T. gondii* infection. Our studies were conducted 3 yr after toxoplasmosis had been controlled with antimicrobial agents, and, moreover, the patient’s T cells exhibited normal proliferation in response to mitogens (22). It appears unlikely that genetic factors, rather than the absence of functional CD40L, were responsible for the pattern of response by T cells from the patient with HIGM syndrome. Studies conducted using TCR-αβ+ and/or unseparated T cells have indicated that in vitro reactivity to *T. gondii* by T cells from 10 seronegative and 6 seropositive individuals can be consistently distinguished at low concentrations of *T. gondii* antigenic preparations (Ref. 19, and current study). Thus, our data strongly suggest that CD40L is crucial for in vivo T cell priming against *T. gondii*. In this regard, studies performed in CD40L knockout mice demonstrate that CD40L is required for in vivo priming of T cells to protein Ags (43). Moreover, patients with HIGM syndrome have impaired lymphocyte proliferation in response to *Candida*, tetanus, and diphtheria toxoids (44). Thus, the absence of CD40L would not only affect APC function (i.e., IL-12 production) but would also impair T cell differentiation. These defects may explain the susceptibility of HIGM patients to opportunistic pathogens.

Although signaling through the CD40-CD40L pathway has been shown to be crucial for acquisition of IFN-γ-dependent protective immune response against *Leishmania major* and *L. amazonensis* in mice (11–13), recent reports have casted doubt on whether these results apply to other intracellular pathogens (14, 15). CD40L knockout mice have been found to develop a protective type 1 immune response against *M. tuberculosis* and *H. capsulatum* (14, 15). However, the fact that an increase in incidence of opportunistic infections is the most prominent clinical feature in patients with HIGM syndrome (10) strongly suggests that CD40L plays an important role in the induction of protective immunity against opportunistic pathogens. The reports of invasive and disseminated infections with *M. tuberculosis* and *H. capsulatum* in these patients (10, 45) raise the possibility that cell-mediated immunity in CD40L knockout mice may not fully mimic the immune response against intracellular pathogens as it occurs in patients with HIGM syndrome.

The effects of CD40LT on IL-12 and IFN-γ production by cells from patients with HIGM syndrome stimulated with *T. gondii* further support the importance of the CD40-CD40L pathway during the immune response to the parasite. Moreover, these data suggest that administration of CD40LT may be useful for the treatment of patients with HIGM syndrome and infections with opportunistic pathogens. It remains to be determined whether defective CD40-CD40L signaling is involved in the susceptibility to opportunistic pathogens in patients with other immunodeficiencies. Of potential relevance to the pathogenesis of congenital toxoplasmosis is the report of impaired expression of CD40L by neonatal T cells, as well as fetal T cells, isolated during the third trimester of gestation (46). In addition, it appears that T cell expression of CD40L may be decreased in HIV-infected individuals (47–49). Finally, our data also suggest that IL-12 may be useful as immunotherapy of disorders associated with defective type 1 immune response.

In summary, we demonstrate that, through regulation of IL-12 production and thus, IFN-γ secretion, the CD40-CD40L pathway plays an important role in the modulation of cellular immunity during *T. gondii* infection. The defect in the IL-12/IFN-γ arm of the immune response detected in patients with HIGM syndrome may explain their susceptibility to infections against which a type 1 immune response is required for host protection. Together with the results of our studies on CD28-CD80/CD86 signaling (18), these data indicate that events that occur during the cognate inter-

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