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Toxoplasma gondii-Induced Immune Suppression by Human Peripheral Blood Monocytes: Role of Gamma Interferon

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The ability of *Toxoplasma gondii* to evade the host immune response during primary infection in humans is poorly understood. In murine toxoplasmosis, infected spleen macrophages release soluble factors that mediate a transient immunosuppression, which may allow the parasite to become established. When an enriched population of human monocytes from seronegative individuals was incubated with toxoplasmas in vitro, soluble factors that mediated marked suppression of mitogen-induced lymphocyte DNA synthesis were released. Irradiated tachyzoites that do not undergo replication were sufficient stimuli for near-maximal soluble factor release. Up to 50% of the soluble factor-mediated suppression is attributable to a gamma interferon (IFN-γ)-dependent pathway, and the mediator of the remaining inhibition is neither interleukin-10, transforming growth factor β, prostaglandin E_2, lipoxygenase products, nitric oxide, nor tumor necrosis factor alpha-induced mitochondrial cell-derived reactive oxygen intermediates. IFN-γ also mediates the up-regulation of an antigen-presenting cell phenotype by both infected and uninfected macrophages. However, IFN-γ does not activate macrophages to become toxoplasmacidal; instead, intracellular toxoplasmas replicate and reinfect, eventually lysing the macrophage population. These results suggest that *T. gondii* is able to evade the naive host immune response by induction of soluble immunosuppressive factors that allow the parasite to become established during an acute infection.

Toxoplasma gondii causes a debilitating disease in unborn children of mothers with primary infection and in the severely immunosuppressed, especially those suffering from AIDS (17, 27). In general, a primary toxoplasma infection of an immunocompetent host is asymptomatic. During acute, primary infection, the host immune system does not eradicate the parasite and the parasite disseminates, mainly to the muscle and brain, remaining latent for the life of the host or until the host is severely immunosuppressed. Little is known, for human infections, about the mechanism by which toxoplasmas initially evade the immune response of a naive host to become established. In mice, acute toxoplasmosis causes nonspecific immunosuppression of the proliferative response of spleen cells to mitogens (3, 23, 25, 37) and of the antibody response to specific and nonspecific antigens (19). Macrophages have been implicated in this immunosuppression (6, 38), and recent studies have shown a role for interleukin-10 (IL-10) and macrophage-derived nitric oxide (6, 16, 22). Immunosuppression of mitogen-induced spleen cell proliferation was maximal on day 7 postinfection, and the response returned to normal by day 21 (6, 16). To characterize further the ability of *T. gondii* to suppress and hence evade the immune response in humans, an enriched population of human monocytes was incubated with *T. gondii* and the effect on mitogen-stimulated lymphocyte DNA synthesis was determined in vitro. We report that incubation of enriched human monocytes with *T. gondii* tachyzoites results in release of soluble factors (SF) that markedly inhibit mitogen-induced DNA synthesis by lymphocytes. An important component of this SF-mediated down-regulatory response appears to be gamma interferon (IFN-γ). We suggest that production of SF following tachyzoite infection may result in a transient general immunosuppression of the host immune system, enabling the parasite to establish itself during an acute infection.

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MATERIALS AND METHODS

Reagents. RPMI 1640 was obtained from JRH Biosciences, Lenexa, Kans. Endotoxin-low fetal bovine serum (FBS) (0.5 EU/ml) was from HyClone Laboratories, Inc., Logan, Utah. Minimal essential medium (alpha modification), antibiotic-antimycotic solution, Versene buffer, and *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) were supplied by Gibco Laboratories, Grand Island, N.Y. Ficoll-Hypaque was from Winthrop Laboratories, New York, N.Y. Butylated hydroxyanisole, concanavalin A, *N*-iminoethyl-L-ornithine, indomethacin, nordihydroguiaretic acid, propidium iodide, rotenone, and thenoyltrifluoroacetone were obtained from Sigma Chemical Co., St. Louis, Mo. Gentamicin sulfate was from United States Biochemical Corp., Cleveland, Ohio. *N*-Monomethylarginine was from Calbiochem, La Jolla, Calif. Paraformaldehyde was from Eastman Kodak Co., Rochester, N.Y. Phytohemagglutinin P was obtained from Pharmacia Biotech Inc., Piscataway, N.J. [^{3}H]thymidine was supplied by Amersham Corp., Arlington Heights, Ill. DIFF-QUIK was from Baxter Healthcare Corp., Miami, Fla. VECTASHIELD was supplied by Vector Laboratories, Inc., Burlingame, Calif. A TiterZyme prostaglandin E_2 (PGE_2) enzyme immunoassay kit (sensitivity, 1.5 pg/ml) was provided by PerSeptive Diagnostics, Cambridge, Mass. EASIA kits for human IFN-γ (sensitivity, 0.03 IU/ml) and human IL-10 (sensitivity, 1 pg/ml) were supplied by INCSTAR Corp., Stillwater, Minn.

Antibodies. Rabbit anti-SAG-1 immunoglobulin G (IgG) and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-SAG-1 IgG were made and purified in our laboratory. Anti-human IL-10 (monoclonal antibody [MAb] 19F1) was graciously provided by J. Abrams, DNAX, Palo Alto, Calif. Anti-human IL-10 neutralizing MAb was obtained from R&D Systems, Minneapolis, Minn. Anti-transforming growth factor β1 [TGF-β1], -β2, -β3 MAb was from Genzyme, Cambridge, Mass. Anti-human IFN-γ antiserum was graciously provided by H. M. Shepard, Genentech, Inc., South San Francisco, Calif.

Mouse anti-human monocyte receptor MAbs included those to CD16 (FcγRIII, MAb 3G8), CD32 (FcγRII, MAb IV.3), and CD64 (FcγRI, MAb 32), all graciously provided by Medarex, Inc., Annandale, N.J.; and to CD11a (LFA-1, MAb TS 1/22.1.1.13.3; hybridoma obtained from American Type Culture Collection [ATCC], Rockville, Md.), CD11b (CR3, MAb OKM1; hybridoma obtained from ATCC), CD11c (p150,95, MAb L29; graciously provided by L. Lanier), CD14 (MAb AML-2-23; graciously provided by M. Fanger), CD18 (β2 integrin subunit, MAb TS 1/18.1.2.11; hybridoma obtained from ATCC), CD29 (β1 integrin subunit, MAb A-1A5; graciously provided by M. Hemler), CD40 (MAb G28-5; graciously provided by R. Noelle), CD54 (ICAM-1; obtained from the Developmental Studies Hybridoma Bank), CD58 (LFA-3, MAb TS 2/9.1.1.4; hybridoma obtained from ATCC), CD85 (B70 [B7/2]; PharMingen, San Diego, Calif.), β_2-microglobulin (MAb BBM1; hybridoma obtained from

ATCC), and HLA-DR, -DP, and -DQ (MAb IVA12; hybridoma obtained from ATCC). MAb P3 (graciously provided by M. Fanger) was used as a negative control. FITC-conjugated $F(ab')_2$ fragments of affinity-isolated anti-mouse IgG was provided by Caltag Laboratories Inc., South San Francisco, Calif.

Monocytes and lymphocytes. Cells were obtained by cytophoresis of healthy volunteers. Mononuclear cells were separated from whole blood with Ficoll-Hypaque, and monocytes were enriched to 80 to 90% by aggregation at 4°C (15). Platelets were removed from monocytes by washing in Versene buffer (0.2 g of EDTA per liter in phosphate-buffered saline). Monocytes were routinely cultured overnight in RPMI 1640 containing 20 mM HEPES, 50 μg of gentamicin sulfate per ml, and 10% heat-inactivated endotoxin-low FBS (medium) at 37°C in tissue culture plates (5×10^5 cells in 500 μl for 24-well plates; 0.8×10^5 cells in 100 μl for 96-well plates) or in Teflon beakers (2×10^6 cells per ml) before infection. The remaining lymphocytes, enriched more than 90%, were cultured in medium (5×10^6 cells per ml) at 37°C in tissue culture flasks until their use in assays 48 h later. They were washed twice immediately before use. Cytocentrifuge preparations of purified monocytes and lymphocytes were stained with DIFF-QUIK, and the percentages of lymphocytes, monocytes, and neutrophils/eosinophils were determined by microscopy. Special care was taken to ensure lipopolysaccharide-free conditions in all of the experiments.

Parasites. *T. gondii* tachyzoites were passaged in human fibroblasts maintained in minimal essential medium containing antibiotic-antimycotic solution and isolated as described previously (21). Briefly, infected fibroblasts were scraped, forcibly passed through a 27-gauge needle, centrifuged at $50 \times g$ for 4 min to remove large host cell debris, and centrifuged at $900 \times g$ for 10 min to pellet parasites. The RH strain is a cloned line of parasites that has been regularly passaged both in vitro and in vivo (31). The Ptg (PLK) strain is derived from the cloned P strain (Me49) and is regularly passaged in human foreskin fibroblasts. The PtgA, PtgB, and PtgC strains are mutants derived from the Ptg wild-type strain after chemical mutagenesis and selection with MAb to the major parasite surface antigen, SAG-1, as previously described (20).

Toxoplasma pretreatments. In one series of experiments, freshly isolated tachyzoites were opsonized, irradiated, or heat killed and then added to macs (see below). Parasites were opsonized for 30 min at room temperature in medium containing 10 μg of rabbit anti-SAG-1 IgG per ml and used without washing (to prevent parasite aggregation). Parasites in wells of a 24-well plate (2×10^6 parasites in 1 ml of medium per well) were exposed to UV irradiation in the center of a biological safety hood for 60 s. This treatment allowed active penetration, but plaques were not formed when parasites were added to cultured fibroblasts. Parasites were heat killed at 56°C for 30 min (heat-killed parasites did not penetrate host fibroblasts). Parasites were added to macs within 2 h of isolation from fibroblasts.

Lymphocyte assay. To determine whether tachyzoite infection resulted in suppression of an immune response, the proliferative response of lymphocytes was assayed in the presence of macs (enriched monocytes cultured for 24 h or longer). Macs represent an initial population of 80 to 90% monocytes that differentiate into monocyte-derived macrophages in culture. Freshly isolated macs were routinely cultured overnight and then incubated for 24 h either with medium (control macs) or with tachyzoites (infected macs). Extracellular toxoplasmas were not washed off, since macs remained nonadherent throughout each experiment. It has been our experience and that of others (29) that toxoplasmas quickly lose their ability to actively penetrate host cells within a few hours and therefore that internalization of added tachyzoites by active penetration would be expected to decline precipitously with time in culture. Lymphocytes/antigen-presenting cells (APCs) (10×10^5 cells for 24-well plates; 2×10^5 cells for 96-well plates) and mitogen (2 μg of phytohemagglutinin P or concanavalin A per ml) were added to macs. Since naive lymphocytes respond poorly to specific antigen, the proliferative response of lymphocytes to mitogen was examined. The concentration of mitogen that induced optimal lymphocyte proliferation was determined in preliminary experiments. To provide the requisite APCs for this response, uninfected macs (not to be confused with control macs) were routinely added to lymphocytes on the day of use to a final concentration of 15%. After a 24-h incubation with mitogen, cultures were pulsed a further 24 h with [^{3}H]thymidine (2 μCi per well for 24-well plates; 0.5 μCi per well for 96-well plates). Cells were harvested on a glass filter with an automated cell harvester, and the isotope incorporation was measured by liquid scintillation counting. Results from three replicate cultures are expressed as mean kilocounts per minute ± standard deviation. In some experiments, the percent inhibition of lymphocyte DNA synthesis was calculated by the following equation: Percent inhibition = $\{1 - [(\Delta\text{cpm of lymphocytes/APCs incubated with infected macs})/(\Delta\text{cpm of lymphocytes/APCs incubated with control macs})]\} \times 100$, where Δcpm is the mean cpm in response to mitogen minus the mean cpm for unstimulated cultures.

To prevent direct cell-to-cell contact during coculture studies, freshly isolated monocytes (5×10^5 in 100 μl of medium) were incubated overnight in 0.4-μm-pore-size, 6.5-mm-diameter tissue culture-treated transwell (TW) inserts (Costar Corp., Cambridge, Mass.) placed in 24-well plates containing 600 μl of medium beneath each TW. Parasites, or medium, were added to TWs for 24 h, and TW inserts were transferred to fresh wells containing lymphocytes/APCs before the addition of mitogen. In this way, the cells in the TW insert do not make contact with the cells in the well of the tissue culture plate into which the insert is placed, although the medium is freely diffusible throughout the TW and the well.

Microscopy. Freshly isolated monocytes (5×10^5/500 μl) were incubated overnight in medium containing 10% FBS in 24-well plates. Under these conditions, the cells remain nonadherent. Parasites were incubated with macs for 2, 24, or 48 h; then the plates were placed on ice, and FITC-conjugated anti-SAG-1 (10 μg/ml) was added for 20 min. Cytocentrifuge preparations were made (70,000 cells per 500 μl; 700 rpm for 5 min in a Cytospin 3 [Shandon Lipshaw, Pittsburgh, Pa.]), and slides were fixed with methanol and then incubated for 5 min at room temperature with 0.5 μg of propidium iodide per ml. An anti-quenching agent was added, and a coverslip was applied. Preparations were examined at ×1,000 magnification in a Zeiss Axiophot microscope equipped for simultaneous observation of red/green fluorescence. In these preparations, the cell surface of extracellular parasites stains green and host cell and parasite nuclei (both intracellular and extracellular parasites) stain red.

Adherence versus nonadherence. When 500,000 freshly isolated enriched monocytes in medium containing 10% FBS were added to a well or to a glass coverslip in a well, they did not adhere and are referred to as nonadherent macs. When the same population of macs was resuspended in serum-free medium at a lower concentration, they became adherent. The largest number of cells that could be added so that more than 90% remained adherent was 200,000 macs per 12-mm-diameter glass coverslip. Hence, freshly isolated diluted enriched monocytes in serum-free medium were added in a 100-μl drop on top of a 12-mm-diameter glass coverslip in a well. After a 30-min incubation at 37°C, 400 μl of medium containing 12.5% FBS was added. For nonadherence, freshly isolated enriched monocytes in 500 μl of medium containing 10% FBS were diluted and added to wells. After overnight culture, all diluted adherent or nonadherent macs were infected with 250,000 Ptg strain tachyzoites. At 2 h after infection, cytocentrifuge preparations of nonadherent cells were made and counted and coverslips containing adherent cells were washed, fixed, and counted. The same number of lymphocytes/APCs with or without mitogen were added to different numbers of either control macs or macs infected for 24 h, and DNA synthesis was measured 48 h later.

Cytofluorography. Freshly isolated, enriched monocytes were incubated overnight in medium in Teflon beakers before the addition of tachyzoites (at a mac/Ptg ratio of 2:1), medium, or IFN-γ for 24 h. Macs were stained with MAbs as described previously (14) and analyzed in a Facscan flow cytometer (Becton Dickinson, San Jose, Calif.). Briefly, macs were removed from Teflon beakers after incubation on ice for 20 min and washed, and 2×10^5 macs in 20 μl were mixed with 20 μl of human IgG (to block nonspecific binding of MAbs to FcγRI) and 20 μl of MAb for 45 min at 4°C. After they were washed, 40 μl of a 1:40 dilution of FITC-conjugated $F(ab')_2$ fragments of affinity-isolated anti-mouse IgG (Caltag Laboratories Inc.) was added for a further 45 min at 4°C. Cells were washed and fixed overnight in 1% paraformaldehyde. CD14-positive cells were gated, and data were acquired with LYSYS software (Becton Dickinson) and analyzed with Cell-Quest software (Becton Dickinson).

Statistics. Statistics for cpm data were analyzed by either one- or two-way analysis of variance (ANOVA) with log-transformed data (10).

RESULTS

Contact between *T. gondii* and enriched monocytes triggers the release of an SF that suppresses mitogen-stimulated lymphocyte DNA synthesis. To determine whether infected human macs can affect mitogen-induced lymphocyte DNA synthesis, macs (enriched monocytes cultured for 24 h) were incubated with *T. gondii* (infected macs) or with medium (control macs) for 24 h and then added to the lymphocyte assay mixture. Figure 1 shows a vigorous mitogen-induced lymphocyte response in the presence of control macs that was inhibited by 85% in the presence of infected macs (compare a and b). Lymphocyte DNA synthesis in the absence of mitogen stimulation, perhaps as a result of FBS components in the medium, was also inhibited in the presence of parasite-treated macs. Similar inhibition of mitogen-induced lymphocyte DNA synthesis was observed when a 0.4-μm-pore-size TW separated lymphocytes/APCs from parasites and macs (Fig. 1, compare c and d), suggesting that an SF that mediates inhibition of mitogen-induced lymphocyte DNA synthesis is released by infected macs.

In a parallel assay, contaminating fibroblast debris prepared in equal concentration and volume did not inhibit the lymphocyte response to mitogen (data not shown). Inhibition is not due to lymphocyte/APC death as a consequence of cell lysis by parasites, since cell numbers and viability were similar for mitogen-stimulated lymphocytes/APCs in the presence of control and infected macs (data not shown) and since a similar

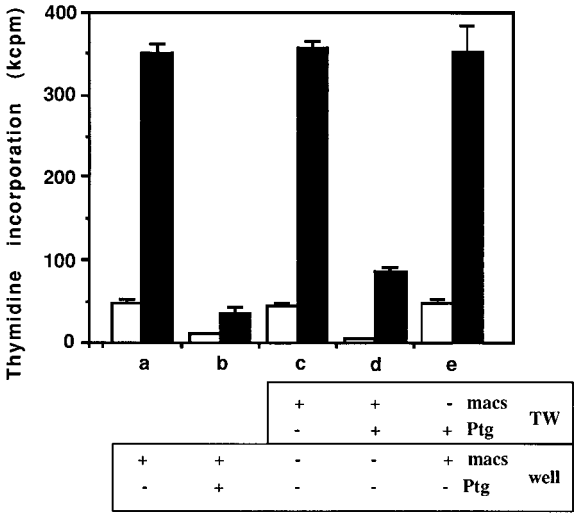

FIG. 1. Contact between untreated parasites and macs triggers the release of an SF that suppresses mitogen-induced lymphocyte DNA synthesis. Medium or Ptg strain tachyzoites at a mac/Ptg ratio of 2:1 were added to macs either in wells or in TW inserts as shown for 24 h, lymphocytes/APCs were added to each well without (□) or with (■) mitogen, and lymphocyte DNA synthesis was measured 48 h later. Values are means ± standard deviations (SD) of triplicate determinations. Results are representative of three donors.

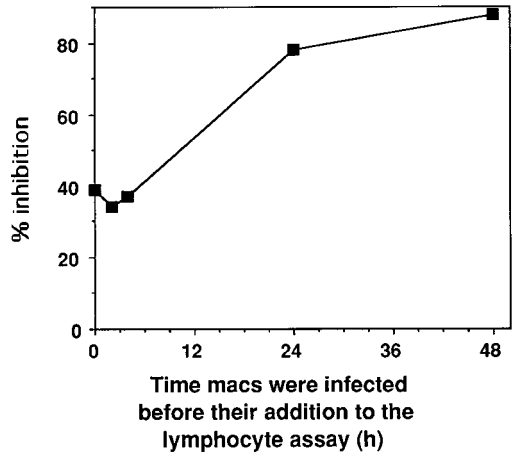

FIG. 2. Maximal inhibition peaks when macs are infected for 4 to 24 h before being added to the lymphocyte assay mixture. Medium or Ptg strain tachyzoites at a mac/Ptg ratio of 5:1 were added to macs in TWs for 2, 4, 24, or 48 h. TWs were transferred to wells containing lymphocytes/APCs, mitogen was added, and DNA synthesis was measured 48 h later. Values were determined from means ± SD of triplicate determinations. Results are representative of two donors.

inhibition of mitogen-induced lymphocyte DNA synthesis was observed through the transmembrane pore that separated lymphocytes/APCs from the parasites and macs.

To determine whether extracellular parasites can release SF, parasites alone were added to TWs with macs in the wells beneath. After 24 h, lymphocytes and mitogen were added to wells and DNA synthesis was determined by thymidine incorporation. When parasites were separated from macs by a TW, there was no inhibition of lymphocyte DNA synthesis (Fig. 1, compare a and e). A similar result was obtained when the number of parasites was increased 10-fold (data not shown). These results suggest that the inhibitory SF is not produced unless parasites are in contact with macs.

Early phase of SF release from infected macs. To determine the time required for contact of macs and parasites to result in maximal inhibition of lymphocyte DNA synthesis, macs and parasites were incubated together in TWs for various times, TWs were transferred to wells containing lymphocytes/APCs, mitogen was added, and DNA synthesis was measured 48 h later. When parasites were added to macs at the same time as lymphocytes/APCs and mitogen, there was 40% inhibition of lymphocyte DNA synthesis (Fig. 2). No further release of SF occurred for 4 h after infection. The highest rate of SF release occurred between 4 and 24 h postinfection, and SF release continued at a lower rate between 24 and 48 h postinfection. The conditioned medium remaining after removal of the TWs was also assayed, and no significant release of SF was found during the 24 h after infection (data not shown). Hence, early release of SF from infected macs peaks between 4 and 24 h after contact of parasites with macs.

Live, nonreplicating parasites are sufficient triggers for SF release from infected macs. To determine the conditions under which *T. gondii* triggers SF release, parasites were treated in various ways before they were added to macs for 24 h. Mitogen-induced lymphocyte DNA synthesis was determined in the presence of control and infected macs, and infection of macs in the absence of mitogen and added lymphocytes/APCs was monitored microscopically over 48 h. No infected lymphocytes were present in the mac population at any time after infection, although an occasional infected neutrophil was observed. Inhibition was similar whether macs were in contact with lymphocytes/APCs or separated from them by TWs.

After a 2-h incubation with either untreated, opsonized, or irradiated tachyzoites (mac/Ptg ratio, 5:1), up to 4% of macrophages became infected while 1% of macrophages internalized heat-killed parasites (Table 1). Digested heat-killed parasites did not significantly inhibit lymphocyte DNA synthesis. Untreated and opsonized parasites were able to replicate, although reinfection was higher for untreated than for opsonized

TABLE 1. Response of macs to infection with toxoplasmas[a]

Parasite pretreatment	% inhibition[b]	Infection time (h)	% of macs infected	No. of toxoplasmas /infected mac	No. of toxoplasmas /100 macs
None	98[c]	2	3	1.4	4
		24	8	2.2	16
		48	39[d]	3.4	131
IgG opsonized	100[c]	2	4	1.2	5
		24	6	3.5	20
		48	13	2.2	29
Irradiated	71[c]	2	3	1.2	3
		24	4	1.5	6
		48	3	1.2	3
Heat killed	15[e]	2	1	1.8	1
		24	0	0	0
		48	0	0	0

[a] Results are representative of two donors.

[b] Numbers represent percent inhibition of the response measured in the presence of control macs (157 kcpm). The uptake of lymphocytes cultured in the absence of mitogen ranged from 14 to 18 kcpm in the presence of control macs or macs treated with heat-killed or irradiated parasites and was 6 kcpm in the presence of macs incubated with untreated or opsonized parasites.

[c] $P < 0.001$ versus control macs by a two-way ANOVA with log data.

[d] Within a further 24 to 48 h of infection, all macs lysed, leaving large numbers of extracellular parasites.

[e] Not significantly different from control macs by a two-way ANOVA with log data ($P < 0.4$).

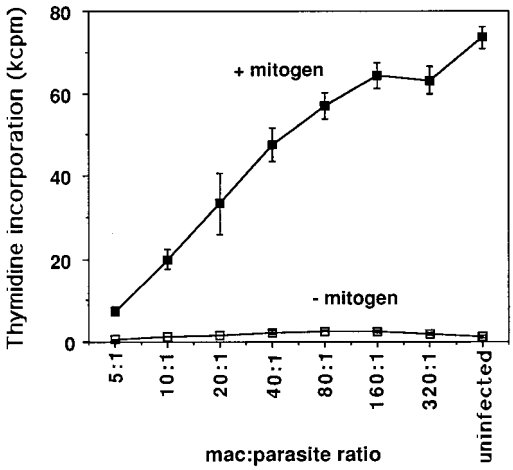

FIG. 3. Inhibition of lymphocyte DNA synthesis is parasite dose dependent. Medium or different numbers of untreated RH strain tachyzoites were added to the same number of macs in wells for 24 h. Lymphocytes/APCs without (□) or with (■) mitogen were added, and DNA synthesis was measured 48 h later. Values are means ± SD of triplicate determinations. Data are representative of three donors.

parasites. Near-100% inhibition of lymphocyte DNA synthesis was observed with either untreated parasites or opsonized parasites. However, irradiated parasites that failed to replicate were sufficient stimuli to result in 71% inhibition of lymphocyte proliferation as determined by DNA synthesis. These results suggest that nonreplicating, live parasites are sufficient triggers for SF release from macs.

SF release is parasite dose dependent. To determine the relationship between parasite number and immunosuppression, a constant number of macs in wells was incubated with increasing numbers of tachyzoites for 24 h before addition to the lymphocyte assay mixture. As shown in Fig. 3, 90% inhibition was seen with a mac/parasite ratio of 5:1 and 50% inhibition was achieved with a ratio of 20:1. A similar inhibitory dose-dependent response was seen for *T. gondii* Ptg and strains deficient in SAG-1 (PtgA, PtgB, and PtgC), suggesting that inhibition does not correlate with expression of the major surface antigen of *T. gondii* (data not shown).

Nonadherent macs release more SF than adherent macs when infected with toxoplasmas, and this correlates with parasite index. The experiments described so far were performed with nonadherent macs. This population of cells routinely comprised 90% monocyte-derived macrophages, 5% neutrophils, and 5% lymphocytes (containing 15% NK cells). The relationship between mac number and immunosuppression and the effect on immunosuppression of adhering the monocytes were then determined. Diluted nonadherent or adherent macs from the same population of cells and differing by an adherence step were cultured overnight, infected with the same number of tachyzoites for 24 h, and added to the lymphocyte assay mixture. The initial infection of diluted macs in the absence of mitogen and added lymphocytes/APCs was also determined microscopically. Since contact between parasites and macs triggers the release of SF, a parasite index was used to estimate the total interaction.

When adherent macs were diluted, decreasing inhibition correlated with both decreasing numbers of macs and decreasing parasite index (Table 2). When nonadherent macs were diluted up to 10-fold, there was no significant change in inhibition, although the parasite index decreased 5-fold (Table 2).

TABLE 2. Inhibition correlates with parasite index and is greater for nonadherent than for adherent macs[a]

Type of mac	No. of macs per well	% Inhibition[b]	Parasite index[c]
Adherent	200,000	78	31,000
	150,000	69	14,000
	100,000	53	13,000
	50,000	37	10,000
Nonadherent	500,000	88	94,000
	250,000	92	66,000
	100,000	85	30,000
	50,000	79	18,000

[a] Results are representative of two donors.
[b] Numbers represent the percent inhibition of the response measured in the presence of control macs (range, 96 to 135 kcpm) and are all statistically significantly different from those for the respective control macs ($P < 0.001$ by a two-way ANOVA with log data). The uptake of [^{3}H]thymidine by lymphocytes cultured in the absence of mitogen ranged from 7 to 14 kcpm in the presence of control macs and from 3 to 14 kcpm in the presence of infected macs.
[c] Parasite index = (mean number of parasites/infected mac) × percentage of macs infected × total number of macs.

Nonadherent infected macs inhibited the lymphocyte response to mitogen significantly more than the same number of adherent infected macs did. For example, with 50,000 macs, both inhibition and parasite index were at least 50% lower for adherent macs than for the same number of nonadherent cells (Table 2). These results suggest that nonadherent macs are more permissive to parasite attachment and invasion and hence are triggered to release more SF than are adherent macs.

Up-regulation of human macrophage receptors during acute infection in vitro. Macrophages play a major role in infection as APCs, presenting antigen together with the appropriate costimulatory surface molecules. To determine whether SF may comprise cytokines that may alter the expression of costimulatory or adhesion molecules on human monocytes, macs were incubated with medium or tachyzoites overnight. As determined by reactivity with anti-receptor antibody and fluorescence-activated cell sorter analysis, receptor up-regulation began 7 h after infection (data not shown). After 24 h of infection, the time at which SF release has peaked (Fig. 2), a two- to fivefold increase in ICAM-1, CD40, and Fcγ RI cell surface expression was observed for infected macrophages compared with uninfected macrophages, although either no change, or less than a 2-fold change, was seen in expression of B7-2, major histocompatibility complex class II and class I, CD29, LFA-3, CD18, CD11a, CD11b, CD11c, or Fcγ RII (Fig. 4 and data not shown). Of note, after 24 h of incubation, with less than 40% of macrophages infected (three toxoplasmas/infected macrophage), a unimodal distribution of fluorescence showed that the entire population of macrophages, both uninfected and infected, modulated their cell surface molecules (e.g., ICAM-1 [Fig. 4]).

IFN-γ mediates up-regulation of macrophage receptors associated with an APC phenotype. The above results indicate that up-regulation of macrophage cell surface receptors in response to infection occurs in both the uninfected and infected macrophages of a population of infected cells. This would suggest that an SF released following infection of host cells may mediate this response. To determine if IFN-γ is released following infection of macs, receptor modulation of uninfected, infected, and IFN-γ-primed macrophages was compared in the presence of anti-IFN-γ or a control antibody. Figure 4 shows that up-regulation of the expression of Fcγ RI,

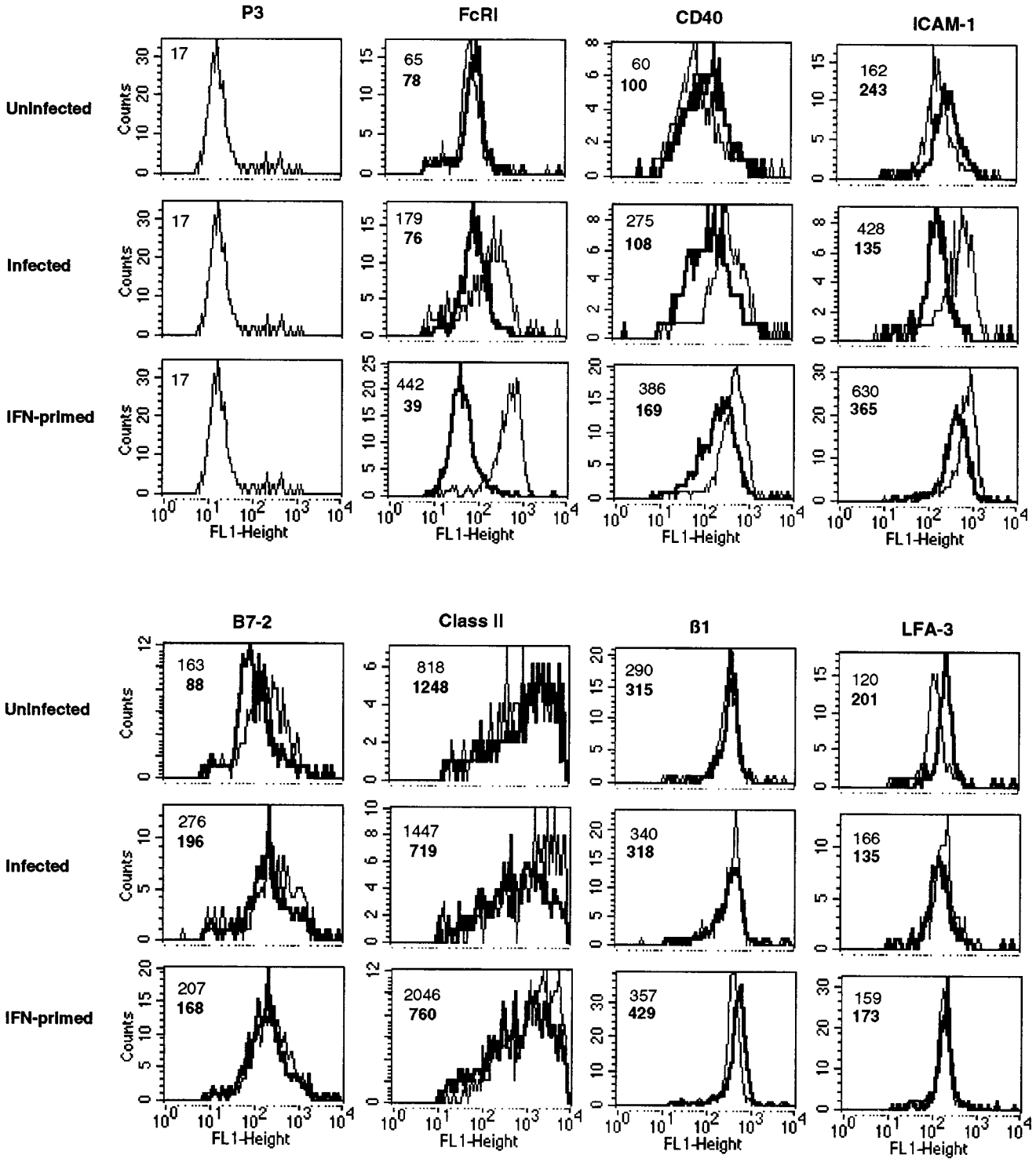

FIG. 4. Effect of anti-IFN-γ on the up-regulation of macrophage receptors following infection or IFN-γ-priming of macs for 24 h. Macs were incubated in Teflon beakers overnight with medium (Uninfected), *T. gondii* (Infected; mac/Ptg ratio, 2:1), or IFN-γ (IFN-primed; 50 IU/ml) in the presence of normal rabbit serum (light lines) or rabbit anti-IFN-γ serum (heavy lines). Cells were stained with anti-receptor antibodies followed by a FITC-conjugated second antibody, and CD14-positive cells were examined by flow cytometry. Graphs represent equivalent numbers of cells. Numbers represent the mean fluorescence intensity of staining for each anti-receptor MAb in the presence of normal rabbit serum or rabbit anti-IFN-γ serum. Results are representative of three donors.

CD40, B7-2, ICAM-1, and major histocompatibility complex class II by macrophages after 24 h of incubation with parasites was ablated in the presence of anti-IFN-γ, whereas CD29 and LFA-3 showed less than a twofold change in expression in the presence of anti-IFN-γ. Similarly, IFN-γ treatment of macrophages resulted in up-regulation of the same subset of receptors, although at a higher level of expression than occurred when parasites were used. Again, this increased expression was

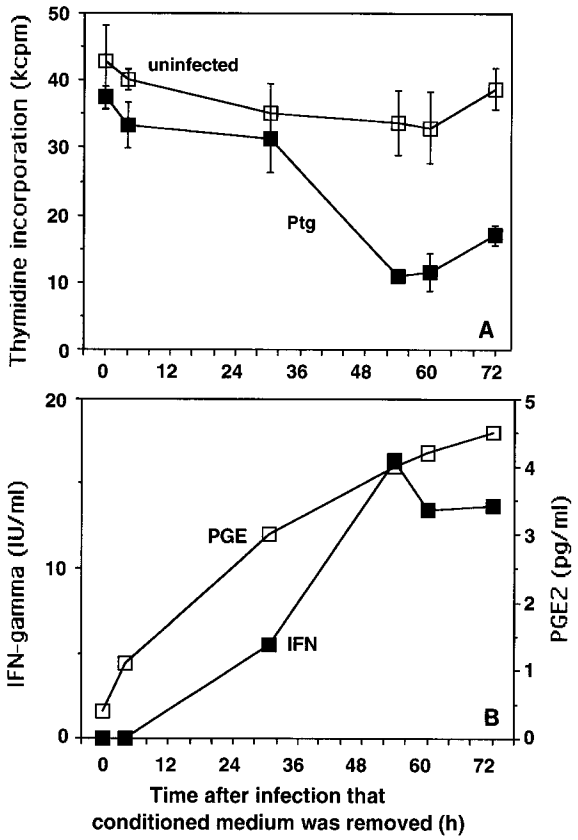

FIG. 5. Kinetics of release of SF, IFN-γ, and PGE_2 from infected macs. Conditioned medium was removed from macs at each time point and centrifuged at 900 × *g* for 10 min, and the supernatant was added either to the lymphocyte assay mixture to determine the presence of SF (A) or to ELISA plates to determine the presence of IFN-γ and PGE_2 (B). Uninfected macs did not release measurable IFN-γ or PGE_2. Values are means ± SD of triplicate determinations. Data are representative of two donors.

ablated in the presence of anti-IFN-γ, with the exception of ICAM-1, for which the expression was reduced rather than abrogated. These results suggest that IFN-γ is produced during the 24-h incubation of nonadherent macs with tachyzoites and that this cytokine mediates up-regulation of a subset of cell surface receptors on all macrophages, both uninfected and infected.

Parasites trigger the increased release of IFN-γ and PGE_2, but not IL-10. Since IFN-γ is produced when macs are incubated with tachyzoites, it is possible that it plays a role in inhibition of mitogen-induced lymphocyte DNA synthesis (2, 7, 9). To determine whether the kinetics of release of SF and IFN-γ were similar, macs were infected and conditioned medium was removed at various times after infection and assayed for SF and for IFN-γ. The greatest release of SF into conditioned medium occurred between 30 and 54 h after incubation of host cells with tachyzoites (Fig. 5). Conditioned medium was also tested by enzyme-linked immunosorbent assay (ELISA) for IL-10 and PGE_2. IL-10 was released in similar small amounts in conditioned medium from host cells incubated in either the presence or absence of parasites. However, IFN-γ and PGE_2 were released only from host cells incubated with parasites, with kinetics similar to SF release (Fig. 5).

To determine whether PGE_2, lipoxygenase products, nitric oxide, TGF-β, IL-10, or tumor necrosis factor alpha-induced mitochondrial cell-derived reactive oxygen intermediates (4, 34) are components of SF, either inhibitors or neutralizing MAbs were added to macs before the addition of parasites and, in some experiments, again before the addition of lymphocytes and mitogen. Indomethacin and nordihydroguiaretic acid (both alone and together), *N*-monomethylarginine, *N*-iminoethyl-L-ornithine, the inhibitors of mitochondrial electron transport (rotenone, butylated hydroxyanisole, and thenoyltrifluoroacetone), anti-TGF-β, and anti-IL-10 did not alter the inhibitory response seen when macs were incubated with parasites (Table 3). Hence, PGE_2, lipoxygenase products, nitric oxide, TGF-β, IL-10, and tumor necrosis factor alpha-induced mitochondrial cell-derived reactive oxygen intermediates are not components of SF.

TABLE 3. Summary of the effect of inhibitors and neutralizing MAbs on mitogen-induced lymphocyte DNA synthesis

Addition to lymphocyte assay[a]	Inhibition	Final concns
Control macs	None	
Infected macs	Maximal	
+ indomethacin	Maximal	0.1, 1, 10, and 100 μM
+ NDGA[b]	Maximal	1, 10, and 50 μM
+ NDGA + indomethacin	Maximal	10 μM each
+ *N*-monomethylarginine	Maximal	0.5, 5, 50, and 500 mM
+ *N*-iminoethyl-L-ornithine	Maximal	10 μm and 1 mM
+ anti-hTGF-β	Maximal	1- and 2-fold the ND[c] for 0.5 ng of total TGF-β per ml
+ anti-hIL-10 (MAb 19F1)	Maximal	10, 50, and 100 μg/ml
+ anti-hIL-10	Maximal	1-, 10-, 100-, and 1,000-fold the ND_{50} of 5 ng of rhIL-10 per ml
+ rotenone	Maximal	2.5, 5, 10, 20, and 40 μM
+ butylated hydroxyanisole	Maximal	25, 50, 100, 200, and 400 μM
+ thenoyltrifluoroacetone	Maximal	25, 50, 100, 200, and 400 μM
+ anti-hIFN-γ	50% of maximal	ND_{100}
IFN-γ	None	0.05, 0.5, 5, and 50 IU/ml
54-h conditioned medium	67% of maximal	1:3 dilution
+ anti-hIFN-γ	67% of maximal	1:3 dilution
IFN-γ-primed macs	40% of maximal	100 IU/ml

[a] Inhibitors or antibodies were added to macs in wells 60 min before the addition of parasites at a 5:1 mac/Ptg ratio for a further 24 h. The concentration of inhibitors or antibodies used was not inhibitory to the lymphocyte assay in the presence of control macs.
[b] NDGA, nordihydroguiaretic acid.
[c] ND, neutralizing dose.

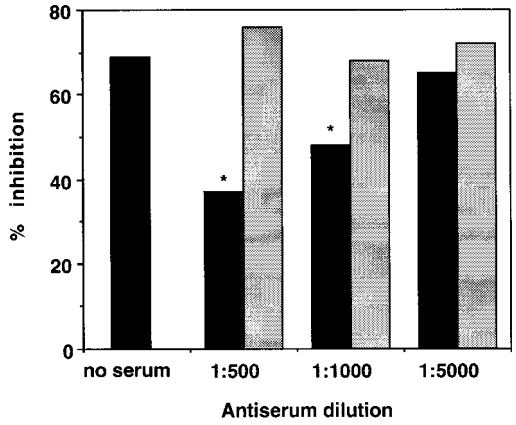

FIG. 6. Effect of anti-IFN-γ on immunosuppression of lymphocyte DNA synthesis. Rabbit anti-IFN-γ serum (■) or normal rabbit serum (▨), followed by medium or Ptg strain tachyzoites at a mac/Ptg ratio of 5:1, were added to macs in wells for 24 h. Lymphocytes/APCs were added with or without mitogen, and DNA synthesis was measured 48 h later. Inhibition was determined by using means ± SD of triplicate determinations. Results are representative of two donors. *, statistically significant difference compared with nonspecific serum ($P < 0.001$ by a two-way ANOVA with log data).

Anti-IFN-γ partially relieves inhibition of lymphocyte DNA synthesis by infected macs. To determine whether IFN-γ played a role in mediating inhibition of mitogen-induced lymphocyte DNA synthesis, macs were incubated for 24 h with medium or parasites in the presence of anti-IFN-γ or a nonspecific antiserum. Incubation of macs with parasites in the absence of serum resulted in 69% inhibition of lymphocyte DNA synthesis. A dose response in which increasing amounts of antibody correlated with decreasing inhibition of lymphocyte DNA synthesis was seen in the presence of anti-IFN-γ antibody (Fig. 6). The presence of a 100% neutralizing dose of anti-IFN-γ antibody (1:500 dilution) resulted in 37% inhibition of lymphocyte DNA synthesis, a nearly 50% decrease in the immunosuppression observed in the absence of antibody.

Late phase of SF release from infected macs. Two approaches were taken to determine whether exogenous IFN-γ directly inhibits mitogen-induced lymphocyte DNA synthesis. Firstly, lymphocytes/APCs were preincubated with various doses of IFN-γ (50, 5, 0.5, and 0.05 IU/ml) before the addition of mitogen, or conditioned medium was preincubated with anti-IFN-γ before the addition of lymphocytes/APCs and mitogen. No inhibition of thymidine incorporation was observed for either condition (Table 3). To evaluate whether exogenous IFN-γ may substitute for parasites as a trigger of SF release from macs, macs were preincubated with increasing amounts of IFN-γ for 24 h before the addition of lymphocytes/APCs and mitogen. Up to 40% inhibition was observed when IFN-γ-primed macs were added to the lymphocyte assay (Table 3). These results suggest that exogenous IFN-γ alone cannot substitute for SF but, rather, that IFN-γ is a cofactor in the pathway to production of the SF that directly inhibits the lymphocyte response to mitogen. Hence, there is also a late phase to the inhibitory response, and SFs other than IFN-γ play a role in this phase.

DISCUSSION

We have reported several essential observations that further our understanding of *T. gondii* infection in humans. First, we have demonstrated for the first time that *Toxoplasma* infection of a recently derived enriched human monocyte population results in the production of SF that suppress the host proliferative response to mitogen stimulation. Second, the induction of this down-regulatory response requires contact between only a small number of parasites and monocytes. Third, SF release is at least a two-phase response. Fourth (and paradoxically), a principal component of SF is IFN-γ, not other factors more frequently associated with immune down-regulation, such as IL-10, nitric oxide, and TGF-β.

Our studies suggest that there must be contact between host cells and parasites for SF release to occur. A single tachyzoite per 20 macs was sufficient stimulus to mediate 50% inhibition of lymphocyte DNA synthesis, indicating that tachyzoites are potent triggers of this response. When live, untreated tachyzoites were used to infect macs, there was nearly complete inhibition of lymphocyte DNA synthesis. The occurrence of intracellular replication of tachyzoites suggests that parasites entered macs by active penetration rather than by phagocytosis. If phagocytosis had occurred, this would have been followed by endosome fusion and acidification, resulting in tachyzoite death (8, 18, 29, 36). Hence, maximal SF release appears to correlate with active penetration. Nearly complete inhibition of lymphocyte DNA synthesis was also observed for macs incubated with opsonized parasites, but this maximal SF release could have been triggered by opsonized parasites that entered by active penetration rather than by phagocytosis (11). Hence, antibody opsonization of the parasite did not allow us to determine whether the mechanism of invasion plays a role in subsequent SF release. The studies with irradiated or heat-killed tachyzoites demonstrated that irradiated parasites could trigger sufficient SF release to mediate 71% inhibition of the proliferative response despite stasis and parasite degradation. In contrast, heat-killed parasites that were phagocytosed in smaller numbers and digested within 24 h failed to trigger significant SF release from macs, presumably because of heat denaturation of triggering antigens. Thus, early SF production, which peaks between 4 and 24 h postinfection (Fig. 2), appears to be triggered by contact between macs and live but not heat-killed parasites and is independent of parasite replication. Hence, products released from macrophages when daughter *T. gondii* cells lyse out are unlikely to mediate inhibition of DNA synthesis by lymphocytes. These conditions that we have described, which result in optimal triggering of SF, are similar to those reported for triggering of IFN-γ release from *T. gondii*-infected spleen cells from *scid* mice (35).

When parasites were incubated with adherent rather than nonadherent macs, there was less inhibition of mitogen-induced lymphocyte DNA synthesis, reflecting less SF release. The larger the number of parasite-host cell triggering events, measured as the parasite index, the greater the inhibition, until maximal inhibition is achieved, and thereafter increasing the parasite index may simply result in a large excess of released SF. Since the monocyte-derived macrophages are the only cells that are infected whether the starting population is adherent or nonadherent, it is plausible that the pathway leading to SF release is triggered when parasites interact with these cells. SF release may be triggered by a specific receptor-ligand interaction between monocyte-derived macrophages and toxoplasmas. To date, the only ligand reported to be involved in this interaction is host-derived laminin, which coats the parasite (12). Since laminin is part of the host extracellular matrix, it is unlikely to be a trigger of SF release. The major parasite cell surface antigen, SAG-1, is also unlikely to trigger SF release, since incubation of macs with either wild-type strains or mutant strains with deficiencies in SAG-1 resulted in similar immunosuppression. Thus, the receptor-ligand interaction that

occurs during infection of human monocytes and triggers SF release remains uncertain.

What does SF comprise? A role for cytokines such as IL-10, which down-regulate B7 or cell-cell adhesion molecules, was considered. Monocyte-derived macrophages modulated their cell surface receptors in response to infection with *T. gondii* concomitant with peak SF release, but there was an increase in the expression of a subset of receptors associated with an antigen-presenting phenotype, a response usually expected as a prelude to eradicating the pathogen. Accessory and cell-cell adhesion molecules either remained at constitutive levels or had increased levels (up to fivefold) in response to infection. Hence, cytokines such as IL-10, which down-regulate B7 or cell-cell adhesion molecules, seem unlikely to be components of the immunosuppressive SF. The up-regulation of monocyte-derived macrophage cell surface receptors, except ICAM-1, following incubation of enriched monocytes with parasites was mediated by IFN-γ. A role for IFN-γ in immunosuppression of lymphocyte DNA synthesis has been reported previously for mice infected with *T. gondii* (7), *Trypanosoma brucei* (9), or *Salmonella typhimurium* (2). When enriched monocytes were incubated for 72 h with parasites in the absence of added lymphocytes and mitogen, a correlation was found between the kinetics of SF release and the release both of IFN-γ and the eicosanoid, PGE_2, suggesting a role for IFN-γ and PGE_2 as mediators of this response. Anti-IFN-γ neutralized nearly 50% of the inhibitory effect of SF derived from infected macs, but PGE_2 was excluded as a mediator, since indomethacin had no effect on the level of inhibition observed. A possible source of this IFN-γ is the residual NK cells in the enriched monocyte population. Our studies also excluded a role for nitric oxide, lipoxygenase products, IL-10, TGF-β, and tumor necrosis factor alpha-induced mitochondrial cell-derived reactive oxygen intermediates in the inhibition of mitogen-induced lymphocyte DNA synthesis. Interestingly, IFN-γ is unable to prime the monocyte-derived macrophages for microbicidal activity, since parasites replicate, reinfect, and eventually lyse the macrophage population. Previous studies have shown that tachyzoites are susceptible to killing by fresh monocytes (26, 39). However, these studies used adherent monocytes washed free of nonadherent cells, whereas our studies used monocytes purified by a cold aggregation step and cultured under endotoxin-free, nonadherent conditions. We have previously shown that nonadherent monocytes are functionally less activated than their adherent counterparts (11).

When does IFN-γ mediate its effect? SF release from infected macs peaked between 4 and 24 h postinfection (Fig. 2). However, when conditioned medium from infected macs was added to the lymphocyte assay, it was not inhibitory until between 30 and 54 h after infection (Fig. 5). These results suggest that SF release has at least two phases, an early phase (4 to 24 h postinfection) resulting in the release of a cofactor that induces the release during a later phase (30 to 50 h postinfection) of a direct mediator of inhibition. Since anti-IFN-γ was without effect when added to conditioned medium removed from infected macs 30 to 50 h after infection, at a time when there was measurable inhibition of lymphocyte DNA synthesis, IFN-γ may be a cofactor involved in the early response. This conclusion is again suggested by the observation that IFN-γ could prime uninfected macs to become inhibitory but could not directly inhibit the lymphocyte assay.

There are striking similarities and differences between the pathways leading to inhibition of mitogen-induced lymphocyte DNA synthesis either by human macs incubated with toxoplasmas in vitro or by infected murine spleen cells taken from animals infected with toxoplasmas. In both systems, toxoplasmas trigger the release of IFN-γ (13), which was reported to mediate completely the inhibitory effect of SF (7). In the murine system, IFN-γ induced the release of nitric oxide, a major mediator of inhibition of lymphoproliferation (1, 6, 22, 28, 32). It is still unclear if human monocytes or macrophages produce nitric oxide (5, 24, 30, 33). Nevertheless, incubation of human macs with parasites in the presence of nitric oxide synthase inhibitors did not relieve inhibition of mitogen-induced lymphocyte DNA synthesis, suggesting that nitric oxide is not a component of SF. A role for IL-10 as an inhibitory mediator was also reported for murine toxoplasmosis (22) but was not seen for these human studies.

In conclusion, our studies suggest that acute *Toxoplasma* infection of human macs triggers the release of a factor(s) that stimulates the release of IFN-γ, which indirectly mediates immune system down-regulation. This down-regulation may be a host response to reduce immune system-mediated tissue damage, to which the opportunistic parasite has adapted in order to establish an infection. It is possible that in a natural infection, once the parasite escapes from the gut of the infected host, it goes on to infect the nonadherent monocytes in the intestinal villi. Infected monocytes may transport the tachyzoites to other sites in the body, e.g., muscle and brain, during the time of maximal SF release. Hence, the host local immune response would be transiently suppressed while the tachyzoite infects tissue cells and transforms into the immunologically cryptic bradyzoite. In humans, acute *Toxoplasma* infection can therefore result in activation of host innate immunity that both protects the host against the sequelae of infection and allows the parasite to persist.

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