Phosphoantigen Presentation by Macrophages to Mycobacterium tuberculosis-Reactive Vγ9Vδ2+ T Cells: Modulation by Chloroquine

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Vγ9Vδ2+ T cells (γδ T cells) are activated by Mycobacterium tuberculosis and recognize mycobacterial nonpeptide phosphoantigens. The role of antigen-presenting cells in the processing and presentation of phosphoantigens to Vγ9Vδ2+ T cells is not understood. We analyzed the role of macrophages for activation of γδ T cells by a new synthetic phosphoantigen bromohydrin pyrophosphate (BrHPP) and M. tuberculosis. Macrophages greatly increased γδ T-cell activation by both BrHPP and M. tuberculosis. Fixation of macrophages before infection demonstrated that uptake of M. tuberculosis was required for presentation to γδ T cells. Antigens of M. tuberculosis remained stably associated with macrophage surface and were not removed by paraformaldehyde fixation or washing. Macrophages processed M. tuberculosis for γδ T cells through a brefeldin A-insensitive pathway, suggesting that transport through the endoplasmic reticulum and Golgi complex of a putative presenting molecule is not important in the early processing of M. tuberculosis antigens for γδ T cells. Processing of M. tuberculosis was not eliminated by chloroquine, indicating that processing of γδ antigens is not dependent on acidic pH in the lysosomes. Chloroquine treatment of BrHPP-pulsed macrophages increased activation of γδ T cells. Ammonium chloride treatment of macrophages did not increase reactivity of γδ T cells to BrHPP, indicating that the effect of chloroquine was independent of pH changes in endosomes. Chloroquine, by inhibiting membrane traffic, may increase association and retention of phosphoantigens with cell surface membrane molecules on macrophages.

Mycobacterium tuberculosis is an intracellular pathogen that infects and resides within mononuclear phagocytes. Cellular immune responses control M. tuberculosis in most healthy individuals, resulting in fewer than 10% of infected persons developing active tuberculosis. T cells and mononuclear phagocytes are required for successful control of M. tuberculosis (11, 29, 37). Mycobacterial antigens are recognized by a variety of T-cell populations, including CD4+ αβ T-cell-receptor-positive (TCR+) T cells (CD4+ T cells), CD8+ αβ TCR- T cells (CD8+ T cells), CD1-dependent double-negative αβ T cells, and γδ TCR+ T cells (γδ T cells) (9, 12, 14, 23, 27). The most common γδ T-cell subset in humans, the Vγ9Vδ2 T cells (γδ T cells), are readily activated by M. tuberculosis (5, 10, 26, 28, 30, 33, 39). Vγ9Vδ2 T cells may serve as a bridge between innate and adaptive immune responses and may have an important role in early immune responses to M. tuberculosis (17). As potent sources of gamma interferon (IFN-γ) and competent cytotoxic effector cells, γδ T cells complement protective functions of CD4+ T cells (6, 20, 25, 42, 52).

Vγ9Vδ2 T cells recognize nonpeptidic compounds with phosphoester structures, collectively called “phosphoantigens.” Among phosphoantigens, TUBags were purified from mycobacterial lysates and found inside M. tuberculosis bacilli (15, 16, 31, 41). They are γ-derivatives of UTP and dTTP (X-γTTP). Other phosphoantigens include pyrophospho- and phosphomonooesters (isopentenyl pyrophosphate [IPP], monothioprophosphate, 2,3 diphosphoglycerate, glyceral 3-phosphate, tuberculosis antigens 1 and 2 [TUBag1-2], and malaria antigens 1 and 2). IPP was isolated from extracts and culture filtrates of fast-growing mycobacteria (M. fortuitum and M. smegmatis) (34, 49–51). IPP is a precursor in cholesterol synthesis and in cholesterol derivatives (steroid hormones, vitamin D, bile salts, and lipoproteins) and terpenoids and is conserved between prokaryotic and eukaryotic cells. The basis of self-nonself discrimination between infected and noninfected cells may consist of recognition of metabolic intermediates (IPP precursors) that are produced through biochemical pathways exclusively present in microbes (Rohmer pathway) (7, 46). The recently identified 3-formyl-1-butyl pyrophosphate is likely a biosynthetic precursor of mycobacterial IPP, eliciting γδ T-cell activation and targeting responses to infected cells (8). This compound is produced in very small amounts in slow-growing mycobacteria such as M. tuberculosis and accumulates to submicromolar concentrations in culture media from fast-growing mycobacterial species. A synthetic analogue called bromohydrin pyrophosphate (BrHPP) has been developed that mimics the biological properties of natural phosphoantigens, is easily synthesized, and is active at nanomolar concentrations (18).

How γδ TCR recognizes phosphoantigens is not fully understood. Phosphoantigen recognition is TCR dependent, re-
quires cell-cell contact, and is not restricted by classical or nonclassical major histocompatibility complex (MHC) (13, 19, 34, 51). Direct binding of γδ TCR to phosphoantigens has not been demonstrated, suggesting the existence of an as, of yet, unknown presenting molecule(s) (31). Phosphoantigens can be recognized without requiring uptake or presentation by professional antigen-presenting cells (APC); however, γδ T-cell responses to intact \textit{M. tuberculosis} bacilli depend on accessory cells (11, 19, 47, 51). Whether accessory cells, in addition to being a source of costimulatory signals, process and/or present phosphoantigens to γδ T cells is not known. γδ T cells can be activated by phosphoantigens directly (in the absence of APC), but it is not clear that this mechanism is important for responses to \textit{M. tuberculosis}. Phosphoantigens of \textit{M. tuberculosis} are found in cytosol of the bacteria and are not secreted. During infection phosphoantigens must traffic from phagosomes to the cell surface by an undefined pathway.

The aim of the present study was to investigate the role of macrophages as APC in the processing and presentation of natural and synthetic phosphoantigens to γδ T cells. \textit{M. tuberculosis} bacilli and IPP were used as sources of natural phosphoantigens, and BrHPP was used as a synthetic analogue. Phosphoantigens and \textit{M. tuberculosis} were compared in terms of dependence on macrophages for γδ T-cell activation. The effect of intracellular antigen-processing inhibitors in early processing of \textit{M. tuberculosis} antigens for γδ T cells was assessed also. Phosphoantigens did not require intracellular processing but remained stably associated on the surface of macrophages in the presence of chloroquine, suggesting an interaction between BrHPP and cell membrane molecules. In addition, intracellularly derived antigens of \textit{M. tuberculosis} were stably associated on the surface of macrophages and were not removed after paraformaldehyde fixation and extensive washings. Our results support a model in which phosphoantigens associate with host molecules on the surface of macrophages and membrane traffic regulates the availability of these phosphoantigens for γδ T-cell recognition.

**MATERIALS AND METHODS**

Monoclonal antibodies and antigens. To identify T-cell subsets, phycoerythrin (PE)-conjugated Leu-4 (CD3-PE), fluorescein isothiocyanate (FITC)-conjugated Leu-3a (CD4-FITC), Leu-2a (CD8-FITC), PE-labeled anti-γδ TCR (clone 11F2), FITC-labeled anti-Vβ2 TCR (clone B6.1), PE-conjugated anti-β2 TCR, and FITC- or PE-conjugated isotypic control antibodies (1) were purchased from BD Pharmingen, San Diego, Calif.

BrHPP was kindly provided by Christian Belmant, Innate Pharma, Marseille, France. IPP was purchased from Sigma Chemical Co, St Louis, Mo. Purified protein derivative (PPD) from \textit{M. tuberculosis} was obtained from Wyeth-Lederle Vaccines, Pearl River, N.Y.

**Bacteria and bacterial lysate.** \textit{M. tuberculosis} H37Ra was cultured in Middlebrook 7H9 with albumin dextrose catabolic enrichment and frozen stocks prepared as described previously (11, 24). Bacterial counts and viability were performed by light microscopy and by counting CFU on 7H10 medium. \textit{M. tuberculosis} H37Ra stocks were tested periodically for viability and with an \textit{M. tuberculosis} complex-specific DNA probe (AccuProbe; Gen-Probe, San Diego, Calif.) to assure purity of \textit{M. tuberculosis} stocks. Before use in T-cell assays, mycobacteria were washed three times in RPMI 1640, sonicated for 40 s, passed multiple times through a 25-gauge needle to disrupt clumps, and diluted in non-heat-inactivated serum-containing medium.

\textit{M. tuberculosis} H37Ra lysate was obtained by sonicating and washing mycobacteria on ice (three 3-min sonications) and then passing them through a French press (three times). Lysate of \textit{M. tuberculosis} was centrifuged for 2 h at 145,000 × g at 4°C. Soluble material was harvested and protein content was determined by Bio-Rad assay (Bio-Rad Laboratories, Richmond, Calif.). Aliquots (5 mg/ml protein) were stored at −80°C. For γδ T-cell stimulation, dilutions of stock material were prepared and normalized according to protein content.

**Isolation of PBMC, monocytes, and macrophages.** Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation over sodium diatrizoate-Hypaque, and monocytes were obtained by adherence from PBMC as previously described (52). PBMC were isolated from healthy tuberculin-positive persons (18 to 45 years old). They were selected for consistency of responses to γδ T-cell expansion (20 to 60% γδ TCR+ T cells) after stimulation with live \textit{M. tuberculosis} for 7 to 10 days.

For preparing monocyte-derived macrophages, PBMC were incubated on plastic tissue culture dishes precoated with pooled human serum; nonadherent cells were removed, and adherent cells were collected by scraping with a plastic policeman. Monocytic cells were resuspended in culture media containing RPMI 1640 supplemented with 10% pooled human serum, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were plated in flat-bottom 96-well plates and allowed to differentiate without addition of growth factors for 7 to 10 days at 37°C.

**Expansion of resting CD4 and γδ T cells by \textit{M. tuberculosis} or phosphoantigen.** PBMC (1 × 10⁶/ml) were cultured with live mycobacteria (2 × 10⁶ or 2 × 10⁵/ml), IPP (10 nM to 1 μM), or BrHPP (1 nM to 1 μM) in a final volume of 6 ml of culture medium. Recombinant interleukin-2 (IL-2) (50 U/ml; Chiron, Emeryville, Calif.) was added after 48 h of culture. After 10 days, cells were harvested and viable cells counted before determining the percentage of CD3+, γδ TCR+, CD4+ and CD8+ T cells by flow cytometry.

**Purification of \textit{M. tuberculosis}-activated CD4+ and γδ T-cell populations.** PBMC stimulated with live \textit{M. tuberculosis} for 10 days were used to obtain CD4+ and γδ T cells by positive selection. Viable cells were harvested by density sedimentation on sodium diatrizoate-Hypaque gradients. CD4+ and γδ T-cell subsets were purified by positive selection with magnetic beads coated with antibodies (Miltenyi Biotec, Gladbach, Germany). For CD4+ T-cell enrichment cells were incubated with beads conjugated to monoclonal mouse anti-human CD4 (Leu-3a). For γδ T-cell purification, cells were first incubated with a hapten-modified anti-γδ TCR antibody, followed by treatment with FITC-conjugated antihapten microbeads. Purity was checked by fluorescence-activated cell sorting. One cycle of selection was sufficient to obtain >95% T-cell purity (by >95% Vδ2-positive cells). Cells were rested for 48 h in medium containing IL-2 (50 U/ml) before use in functional assays.

**Immunofluorescence analysis.** FITC-anti-Vβ2 TCR, PE-anti-pan γδ TCR, FITC-anti-β2 TCR, FITC-anti-CD4, FITC-anti-CD8, and PE-anti-CD3 antibodies were used to assess percentages of γδ TCR+ T cells and αβ TCR+ T cells in unstimulated or stimulated cultures by two-color fluorescence-activated cell sorting. To check the purity of positively selected CD4+ and γδ T cells, PE-anti-CD3 was used with FITC-anti-CD4 and FITC-anti-γδ TCR antibodies.

Cells were analyzed on a FACScan (Becton and Dickinson) with the CellQuest software. Cells were gated in a two-parameter plot of 90% versus forward angle scatter. The gate for lymphocytes or monocytes was set widely. Five thousand events were recorded for each cell surface marker. The cutoff lines for positive and negative fluorescence were set manually based on the distribution of isotypic control antibodies stained with FITC and PE-conjugated isotypic control antibodies alone and were kept constant within each experiment. The percentage reported for a given surface marker represents the proportion of gated cells with a positive signal less the percentage of cells staining positive with isotypic control antibody alone.

**Proliferation and IFN-γ assays.** PBMC or prestimulated and positively selected CD4+ and γδ T cells from different donors (5 × 10⁵ cells per 200 μl-well) were cocultured with or without autologous macrophages or phorbol 12-myristate 13-acetate (Sigma-Aldrich, St Louis, Mo.) differentiated-THP-1 cultures (ATCC TIB-202) as APC (10⁵ cells per 200 μl-well). Cultures were stimulated with \textit{M. tuberculosis} (multiplicity of infection [MOI] = 1:1, 10:1, or 50:1), \textit{M. tuberculosis} lysate (1:50 to 1:400 dilutions), IPP (100 nM to 1 μM), or BrHPP (10 nM to 10 μM) for 72 h in 96-well plates. Cells were pulsed with 1 μCi of [3H]thymidine (ICN, Costa Mesa, Calif.) for 12 to 16 h before harvesting on glass fiber filters. [3H]thymidine incorporation was measured by liquid scintillation counting and expressed as counts per minute. Before pulsing the cultures with [3H]thymidine, 50 μl of supernatant was harvested from each well for measurement of IFN-γ. IFN-γ was measured by sandwich enzyme-linked immunosorbent assay (ELISA) with M70-A and M70-B antibodies (Endogen, Cambridge, Mass.).

**Macrophage antigen presentation assay.** Macrophages were placed in 96-well plates (1.5 × 10⁵ cells/well) and infected with \textit{M. tuberculosis} (MOI = 10:1) or pulsed with \textit{M. tuberculosis} lysate (1:50 dilution = 250 μg of protein/ml) or BrHPP (0.1 to 100 μg/ml). After 4 h, cells were washed with RPMI and incubated with 1% paraformaldehyde for 15 min at room temperature. Plates were washed...
twice with RPMI before addition of 0.2 M lysine. After 20 min, lysine was discarded and plates were washed four times with RPMI. Alternatively, noninfected macrophages were fixed and antigen pulse was performed for 4 h. After infection and pulse were carried out, macrophages were washed extensively and 5 \times 10^6 purified CD4^+ or γδ T cells were added to the wells. On day 3 supernatants were harvested for IFN-γ measurement by ELISA and cells were pulsed with 1 μCi of [3H]thymidine for 12 to 16 h before harvesting and proliferation was determined as described above.

For brefeldin A (BFA) and chloroquine treatment, cells were pretreated with BFA (Boehringer Mannheim Biochemical, Indianapolis, Ind.) at 5 μg/ml, chloroquine (Sigma-Aldrich) at 5 to 500 μM, or ammonium chloride at 5 to 40 mM for 30 min before addition of the antigens. Inhibitors were present during the entire period of incubation (4 h) with antigens. APC cells were washed and fixed with paraformaldehyde, and purified CD4^+ or γδ T cells (5 \times 10^6 cells/well) were added. After 3 days, IFN-γ was measured in culture supernatants and T-cell proliferation was assessed by [3H]thymidine incorporation.

**Statistical analysis.** Statistical analysis was determined by Student’s t test, and a P of <0.05 was considered significant.

**RESULTS**

BrHPP expands γδ T cells from peripheral blood at nanomolar concentrations and requires IL-2 for proliferation and IFN-γ secretion. First the ability of BrHPP to expand resting γδ T cells from peripheral blood was compared to T-cell expansion by IPP and M. tuberculosis. PBMC from healthy individuals (10^6 cells/ml; 6 ml of culture) were stimulated with live *M. tuberculosis* bacilli (1:1 or 10:1 *M. tuberculosis*-to-macrophage ratio), IPP (100 nM to 10 μM), or BrHPP (10 nM to 1 μM) in the presence of IL-2 (50 U/ml). After 10 days, viable cells were harvested, counted, and analyzed by two-color flow cytometry for CD3, CD4, CD8, and γδ TCR expression. Results are expressed as absolute number of γδ TCR^+ T cells at the end of culture (Fig 1A). γδ T cells expanded from 3 to 5% at baseline to 20 to 60% after stimulation with *M. tuberculosis* or phosphoantigens (n = 5). As shown in Fig. 1A, *M. tuberculosis* induced the greatest expansion of γδ T cells after 10 days of stimulation. When IPP and BrHPP were compared, it was observed that BrHPP induced the same degree of γδ expansion as IPP but at 100-fold lower concentrations. While threshold concentrations of these antigens for γδ expansion varied from donor to donor, BrHPP consistently had a lower threshold dose than IPP (i.e., when BrHPP caused a significant γδ T-cell expansion at 100 nM, IPP threshold was 1 μM). Neither BrHPP nor IPP caused expansion of CD4^+ or CD8^+ T cells (data not shown). Responses to BrHPP were completely eliminated by pretreatment with calf intestinal alkaline phosphatase (Promega, Madison, Wis.) confirming that the pyrophosphate was responsible for γδ T-cell activation (data not shown). Thus, BrHPP on a molar basis had greater activating capability for γδ T cells than IPP and thus is suitable for studies of antigen presentation.

As described previously, γδ T-cell activation by natural phosphoantigens (IPP) depends not only on TCR signaling but also on additional signals provided by T-cell growth factors such as IL-2 (53). We confirmed that proliferation and IFN-γ secretion by γδ T cells in response to the synthetic phosphoantigen BrHPP depended on IL-2. Without IL-2 minimal proliferation and no IFN-γ secretion was observed even at high phosphoantigen concentrations (Fig. 1B and C).

**Optimal responses of γδ T-cell lines to BrHPP require APC.** To study the role of APC on γδ T-cell activation, γδ T cells were first expanded from PBMC of different donors with *M.
tuberculosis for 10 days. After positive selection for γδ T cells, highly purified (>95%) Vγ9Vδ2 TCR+ cells were restimulated with M. tuberculosis, M. tuberculosis lysate, or phosphoantigens in the presence or absence of autologous macrophages. Antigen-specific responses (proliferation and IFN-γ secretion) were observed with M. tuberculosis and with BrHPP in the absence of macrophages, but the addition of macrophages increased γδ T-cell responses three- to fourfold (Fig. 2). Thus, although M. tuberculosis and phosphoantigens can somewhat activate γδ T cells directly in absence of macrophages, optimal responses are obtained in the presence of macrophages. Experiments conducted with resting γδ T cells purified from freshly isolated PBMC demonstrated that activation requirements for resting γδ T cells were the same as those observed for in vitro-activated γδ T cells. Antigen-presenting cells greatly increased resting γδ T-cell responses (data not shown).

In Fig. 3 increased γδ T-cell responses in the presence of macrophages was not dependent on matching of MHC, since γδ T-cell lines and macrophages used in these experiments did not share MHC class I (MHC-I) or MHC-II haplotypes. In addition, γδ T-cell line responses to antigen-pulsed matched and mismatched macrophages from different donors were similar, confirming the lack of self MHC restriction (data not shown). Thus, γδ T-cell responses to the new synthetic phosphoantigen BrHPP resemble those observed to natural phosphoantigens or M. tuberculosis bacilli both in terms of the ability of APC to enhance γδ T-cell activation and the lack of self MHC restriction.

Uptake of M. tuberculosis by macrophages generates antigens that are stably presented to γδ T cells. The role of antigen uptake and intracellular processing was first assessed by means of pulse fixation experiments. In these experiments, macrophages were infected or pulsed with antigen before or after fixation with paraformaldehyde. Cells were extensively washed after antigen pulse and therefore there was no free (soluble) antigen present at the time of T-cell addition. As shown in Fig. 4 macrophages fixed after infection with M. tuberculosis were able to activate γδ T cells, although at much lower levels than nonfixed macrophages (Fig. 2A). In contrast macrophages exposed to M. tuberculosis after fixation did not activate γδ T cells. Responses to soluble antigens such as M. tuberculosis lysate and BrHPP pulsed onto the macrophages before fixation were low but detectable. These experiments suggest that uptake of M. tuberculosis and subsequent degra-

![FIG. 2. Accessory cells enhance γδ T-cell responses to M. tuberculosis and phosphoantigens. Prestimulated and positively selected γδ T cells (5 × 10^6 cells/well) from different donors were restimulated with M. tuberculosis (MTB) at the indicated bacterium/cell ratio) or BrHPP (10 nM to 1 μM) in the presence (black bars) or absence (gray bars) of autologous macrophages (10^5 cells/well). (A) Proliferation is expressed as stimulation index (counts per minute of stimulated cultures/counts per minute of unstimulated cultures). (B and C) IFN-γ was measured in 48-h culture supernatants by ELISA. One representative experiment of four is shown.](image-url)
dation inside APC results in antigens for γδ T cells that are stably associated with the cell surface.

Effect of BFA and chloroquine on M. tuberculosis and phosphoantigen processing by human macrophages. To determine if M. tuberculosis and phosphoantigens differ in cellular processing requirements for γδ T-cell activation, macrophages were treated with BFA or chloroquine before (30 min) and during antigen pulse (4 h). BFA treatment had no effect on early processing of M. tuberculosis and did not affect phosphoantigen presentation to γδ T cells (n = 4). Control experiments demonstrated that BFA inhibited MHC-II processing of tetanus toxoid (TT) for a tetanus toxoid-specific hybridoma (data not shown). These results indicated that unlike classical MHC-I or MHC-II antigen-processing pathways, anterograde transport through the endoplasmic reticulum and Golgi had no role in antigen processing for γδ T cells. Chloroquine did not eliminate M. tuberculosis processing and presentation for γδ T cells. Surprisingly, chloroquine treatment of phosphoantigen pulsed-macrophages consistently increased activation of γδ T cells (Fig. 5). These findings suggest either that intralysosomal pH variation by chloroquine does not affect generation of γδ-activating antigens in M. tuberculosis-infected macrophages or that potential down-regulation of processing by chloroquine is obscured by up-regulation of phosphoantigen presentation.

γδ T-cell activation by phosphoantigen-pulsed macrophages is enhanced by chloroquine treatment. To further characterize the chloroquine effect, macrophages were treated with different concentrations of chloroquine (5 μM to 500 μM) for 30 min and pulsed with BrHPP or IPP for 4 h in the continuous presence of chloroquine. Macrophages then were washed, fixed, and used to stimulate γδ T cells. As shown in Fig. 6, even at low concentration of chloroquine (5 μM), BrHPP (100 nM) was able to stably associate with macrophages for presentation to γδ TCR. Macrophages pulsed in the absence of chloroquine needed high concentrations of BrHPP (>10 μM) to stimulate γδ T cells. Chloroquine had the same effect when M. tuberculosis

FIG. 3. Up-regulation by macrophages of γδ T-cell responses is not MHC restricted. Prestimulated and positively selected γδ T cells (5 × 10⁴ cells/well) from different donors were restimulated with M. tuberculosis (MTB) (A), M. tuberculosis lysate (B), BrHPP (C), or IPP (D) in the presence (+ APC) or absence (No APC) of mismatched THP-1 macrophages (10⁵ cells/well). IFN-γ levels in 48-h culture supernatants were determined by ELISA. Shown are the mean values of an experiment representative of three.

FIG. 4. Effect of fixation of macrophages on their ability to present M. tuberculosis and phosphoantigens to γδ T cells. Macrophages (1.5 × 10⁵ cells/well) were fixed before or after M. tuberculosis (MTB) infection (MOI = 10:1), treatment with M. tuberculosis lysate (dilution 1:20), or BrHPP (10 μM) pulse. Antigens were washed away, and macrophages were used to stimulate γδ T-cell lines (5 × 10⁴ cells/well). IFN-γ was measured (ELISA) in 48 h-culture supernatants. Mean values ± standard errors (error bars) of one representative experiment of three are shown.
lisis lysate or the natural phosphoantigen IPP was used as antigen. Up-regulation of phosphoantigen presentation by chloroquine was seen at concentrations of chloroquine higher than 5 μM and at concentrations of BrHPP less than or equal to 10 μM (IPP less than or equal to 100 μM), suggesting a dose-dependent mechanism in which a saturation state is reached at high antigen concentrations.

The effect of chloroquine on processing and presentation of a soluble antigen (PPD) for MHC-II-restricted CD4+ T cells was investigated also. Macrophages from the same donor were treated with chloroquine and used to present antigens to CD4+ or γδ T cells. Figure 7 demonstrates that processing and presentation of PPD, especially at high antigen concentrations, was readily inhibited by chloroquine. In contrast, BrHPP responses were significantly increased when cells were treated with 50 μM chloroquine. Parallel results were obtained when [3H]thymidine incorporation (proliferation) was used as a measure of γδ T-cell or CD4+ T-cell activation (data not shown).

Next, we compared chloroquine with another lysosomotropic agent, ammonium chloride. Figure 8 demonstrates the effect of chloroquine and ammonium chloride treatment on BrHPP-pulsed macrophages. Chloroquine-treated BrHPP-pulsed macrophages exhibited up-regulated stimulatory capability for γδ T cells in a dose-dependent manner compared to non-chloroquine-treated macrophages. In contrast, a wide range of ammonium chloride concentrations (5 to 40 mM) failed to affect γδ T-cell responses to BrHPP, indicating that the effect is independent of pH changes in the lysosomes.

**DISCUSSION**

The most-common γδ T-cell subset in adult humans, Vγ9Vδ2 T cells, can be stimulated by a variety of pathogenic microorganisms (36). The common feature of Vγ9Vδ2 T-cell-stimulating compounds is the presence of phosphate groups. Among these antigens, TUBags and IPP were the first ones to be identified. How phosphoantigens are recognized by γδ TCR is not understood. Although intracellular processing may not be required, cell surface presentation and possibly extracellular processing may play a role in optimal recognition of ligands by γδ TCR (7). However, the existence of presenting molecules and requirements for cell surface processing of phosphoantigens remain to be established.

The present study compared responses to a new synthetic phosphoantigen, BrHPP; the natural phosphoantigen IPP; and M. tuberculosis bacilli—particularly focused on requirements for antigen processing and presentation. First the ability of a new synthetic phosphoantigen, BrHPP, to expand and stimulate Vγ9Vδ2 T cells was investigated. BrHPP was more potent on a molar basis than IPP in stimulation of Vγ9Vδ2 T cells. M. tuberculosis was the most-potent stimulator of γδ T cells compared to either phosphoantigen. Consistent with previous reports, Vγ9Vδ2 T-cell responses to phosphoantigen were dependent on exogenous IL-2 (53).

Our studies demonstrate that γδ T-cell responses to both M. tuberculosis and to soluble antigens were markedly increased in the presence of APC. Most studies have suggested that enhanced γδ T-cell responses in the presence of APC are primarily due to costimulatory activity rather than antigen processing and presentation. The large increases in γδ T-cell responses observed in our study in the presence of APC suggest additional mechanisms besides costimulation, such as intracellular or extracellular processing or association to a receptor or presenting molecule resulting in increased affinity of the
epitope or allowing multivalent interaction with the Vγ9Vδ2 TCR. The APC-enhancing effect on γδ T-cell responses was dependent on APC density (data not shown) and type of APC (i.e., THP-1 increased γδ T-cell responses more than autologous or heterologous macrophages [Fig. 2 and 3]). In addition, responses with live macrophages in continuous presence of the antigen were higher than responses to fixed and/or pulsed macrophages. The fact that γδ T cells can respond to antigens in the absence of macrophages is consistent with a model of T-cell–to–T-cell presentation.

Pulse-fixation experiments demonstrated that viable cells were required for M. tuberculosis presentation to γδ T cells. These results suggest that intact mycobacteria need intracellular processing for phosphoantigens to become available for recognition on the surface of APC. Two inhibitors of intracellular processing were tested: BFA and chloroquine. BFA inhibits transport from the endoplasmic reticulum to the trans-Golgi network (22). BFA blocks the supply of nascent MHC-I and MHC-II molecules to the endogenous and endocytic pathways, respectively, and inhibits the formation of most peptide-MHC complexes in the endoplasmic reticulum or in late endocytic compartment (2). Previous work by our group focused on effects of BFA on ongoing antigen processing (i.e., after 12 h of pulse with the antigen) and used cytotoxicity as a measure of γδ T-cell activation. Our present approach differs kinetically from the previous one in that BFA was used to study initial processing (i.e., occurring at the time of antigen uptake) and γδ T-cell activation was assessed by proliferation and IFN-γ production. In our present work BFA did not inhibit initial processing of M. tuberculosis or soluble phosphoantigens. In contrast, previous studies demonstrated that BFA partially inhibited ongoing processing of M. tuberculosis in unfixed monocytes in a CTL assay (4). This may indicate that a different pool of presenting molecules is used in initial processing which does not involve de novo synthesis and trafficking through endoplasmic reticulum to the trans-Golgi network.

Our present experimental approach indicates that nascent presenting molecules are not required for initial processing and presentation of M. tuberculosis antigens to γδ T cells.

Chloroquine is a weak base amine that inhibits both MHC-II and MHC-I antigen-processing pathways (endosomal processing pathways) (21, 32). Ammonia and weakly basic alkylamines increase the endosomal and lysosomal pH and thus decrease degradation of endocytosed proteins, affecting ligand-receptor dissociation and receptor recycling (40, 45). Some authors have reported that apart from effects on endocytic compartments, certain Golgi functions, i.e., transport of lipoproteins or antibody secretion and/or glycosylation, may be affected by changes in pH caused by chloroquine or ammonia (inhibition of MHC-I processing) (43, 48). The effect of ammonia is primarily due to increased pH, whereas chloroquine has a variety of additional effects. These include direct inhibition of protein-

FIG. 7. Effect of chloroquine on M. tuberculosis and soluble antigen presentation by macrophages to γδ and CD4+ T cells. Macrophages (1.5 × 10⁵ cells/well) were treated with indicated concentration of chloroquine and pulsed with indicated concentrations of M. tuberculosis, BrHPP or PPD for 4 h. Macrophages were washed, fixed and used to stimulate positively selected γδ (A and B) or CD4+ T cells (5 × 10⁴ cells/well) (C). Culture supernatants were collected after 48 h, and ELISA was used to measure IFN-γ. Shown are mean values ± standard errors of a representative experiment.
not use macrophages as APC, but use Epstein-Barr virus-transformed B-cell lines, syngeneic and allogeneic PBMC, B lymphoma cells, fibroblasts, and Vγ9Vδ2 T-cell clones. Furthermore, in these previous reports the APCs were not fixed after antigen pulse. Although the presence of antigen on cell surface has not been formally demonstrated, the ability of phosphoantigen-pulsed chloroquine-treated macrophages to stimulate M. tuberculosis-reactive γδ T cells after extensive washing and fixation suggests that the antigen was in fact stably associated with the surface of the APC and presented to the γδ TCR. The fact that prefixed macrophages could not be pulsed with phosphoantigens indicates that the interaction of antigen with macrophage membrane is less stable than the MHC-peptide interaction (classical MHC presentation of peptides) or that putative presenting molecules become unavailable or unreceptive after fixation.

Recent reports of the human γδ TCR crystal structure and docking of phosphoantigens into a Vγ9Vδ2 model do not provide a mechanism for γδ T-cell activation by direct interaction of small phosphoantigen and the γδ TCR. These studies leave open the possibility of an antigen-presenting molecule (3, 35). Our results present indirect evidence for an interaction between γδ TCR, phosphoantigens, and cell membrane components that promote γδ T-cell activation. Chloroquine inhibition of membrane turnover may allow greater retention of phosphoantigens at the cell surface and hence greater γδ TCR-antigen contact.

In summary BrHPP triggers γδ T-cell responses more efficiently than natural phosphoantigens (IPP), and the response is increased in the presence of APC and chloroquine. Although further studies are required to understand the mechanism, the ability of phosphoantigen-pulsed chloroquine-treated macrophages to stimulate γδ T cells may be an important tool in understanding the interaction between γδ T cells and microbial pathogens such as M. tuberculosis.

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