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Protein-DNA complex is the exclusive malaria parasite component that activates dendritic cells and triggers innate immune responses[1]

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Abstract

DCs play crucial role in the development of protective immunity to malaria. However, how malaria parasites trigger immune responses in DCs remains unclear. Here, we purified merozoites, food vacuoles and parasite membrane fragments released during the *Plasmodium falciparum* schizont burst to homogeneity and tested for the activation of BMDCs from wild type and TLR2$^{-/-}$, TLR4$^{-/-}$, TLR9$^{-/-}$ and MyD88$^{-/-}$ C57BL/6J mice. The results demonstrate that a protein-DNA complex is the exclusive parasite component that activates DCs by a TLR9-dependent pathway to produce inflammatory cytokines. Complex formation with proteins is essential for the entry of parasite DNA into DCs for TLR9 recognition, and thus proteins convert inactive DNA into a potent immunostimulatory molecule. Exogenous cationic polymers, polylysine and chitosan, can impart stimulatory activity to parasite DNA, indicating that complex formation involves ionic interactions. Merozoites and DNA-protein complex could also induced inflammatory cytokine responses in human blood DCs. Hemozoin is neither a TLR9 ligand for DCs nor functions as a carrier of DNA into cells. Further, while TLR9 is critical for DCs to induce the production of IFN-γ by NK cells, this receptor is not required for NK cells to secret IFN-γ and cell-to-cell contact among myeloid DCs, plasmacytoid DCs and NK cells is required for IFN-γ production. Together, these results contribute substantially toward the understanding of malaria parasite recognition mechanisms. More importantly, our findings that proteins and carbohydrate polymers are able to confer stimulatory activity to an otherwise inactive parasite DNA have important implications for the development of vaccine against malaria.

Malaria, caused by the protozoan parasites of the genus *Plasmodium*, is an enormous health problem in many countries of the globe with nearly half of the population is at risk (1,2). Of several *Plasmodium* species that cause malaria in human, *P. falciparum* is the most virulent and is responsible for majority of deaths (3). Despite large research efforts in many laboratories during the past two decades, a strategy for the development of an effective vaccine for malaria is still not available (4,5), pointing to a significant gap in our understanding of the parasite-

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host interaction mechanisms in the development of immunity to malaria. A clear knowledge of the parasite components and the host receptors involved in the immune responses is crucial for gaining a better insight into malaria pathogenesis and protective immunity. This will in-turn broaden our ability to develop strategies for effective malaria treatment.

The erythrocytic stage infection accounts for all of the accompanied pathological conditions of malaria infection. Many of the clinical symptoms of malaria infection, including fever and chills correspond to the production of high levels of pro-inflammatory cytokines such as TNF-α, IL-12, and IFN-γ by the innate immune system in response to the parasite components released during the schizont rupture (6,7). These components are: merozoites (MZs), food vacuoles (FVs) containing hemozoin (HZ) crystals, fragments of parasite membranes, and soluble components released into the blood stream. However, which of these components effectively induce innate immune responses in DCs remain poorly understood.

Innate immunity to parasite plays two important functions. First, it acts as the first line of defense to limit the rapid parasite growth and onset of malaria pathology (5,6). Second, it is crucial for the development of parasite-specific adaptive immunity and shaping up of the malaria protective immunity (8,9). DCs are the important components of the innate and adaptive immune system. They are highly effective in detecting pathogens and play critical roles in the initiation and regulation of immune responses (10,11). At early stages of infection, DCs produce pro-inflammatory cytokines, which control parasite growth and regulate adaptive immune responses, and at later stages produce anti-inflammatory cytokines to avoid pathogenesis (12). Importantly, DCs take up antigens, process, and present them to T and B cells, thereby connecting the innate and adaptive arms of the immune system (13,14).

Toll-like receptor (TLR) proteins are the key molecules of the innate system that target various pathogenic microorganisms to mediate innate immune responses (15,16). Upon recognition of a microbial ligand, the cytoplasmic tail of TLR binds specific adaptor proteins, initiating recruitment of several signaling components, leading to the activation of MAPK and NF-κB signaling cascades and production of cytokines and other immune responses (16,17). The TLR-mediated signaling is known to control antigen uptake, antigen presentation, and DC maturation (17,18).

To-date a clear understanding of the malaria parasite components and molecular interactions involved in their recognition by the innate immune system remain unclear. Glycosylphosphatidylinositols (GPIs) of *P. falciparum* have been shown induce inflammatory cytokine production by macrophages through TLR2 recognition (19). Pichyangkul *et al.* have found that the soluble extracts of *P. falciparum* can stimulate DCs to express costimulatory molecules and cytokines, but the identity of the parasite molecules was not defined (20). Recently hemozoin has been reported to activate DCs through TLR9 recognition (21). However, a subsequent study suggested that hemozoin is immunologically inert, but it is involved in enhancing the activity of parasite DNA to stimulate DCs (22). Thus, the role of hemozoin in DC activation remains doubtful.

In this study, by dissecting the various components of blood stage *P. falciparum*, we show that a protein-DNA complex is the component that activates DCs by engaging TLR9 and this activity confines to MZs among the various parasite components released during the schizont rupture. The cooperation of proteins is absolutely essential for the stimulatory activity of otherwise inactive DNA. Further, we show that a contact-dependent cooperation between myeloid DCs (mDCs), plasmacytoid DCs (pDCs), and NK cells is required for the production of IFN-γ by NK cells, and for this response TLR9 expression in NK cells is not required. Additionally, our results demonstrate that hemozoin has no role in the TLR9-mediated stimulatory activity of the parasite DNA in DCs. Since DCs play critical role in the development

and modulation of protective immunity to malaria, our data have important implications for the development of an effective vaccine against malaria.

Materials and Methods

Reagents

CpG ODN-1826 was from Coley Pharmaceutical Canada (Kanata, ON, Canada). CpG ODN-2006 was from Cell Sciences (Canton, MA). Pam_3CSK_4 was from Microcollections (Tübingen, Germany). LPS from *Salmonella minnesota* Re595 strain (catalog # L9764), sodium pyruvate, 2-mercaptoethanol, poly-L-lysine hydrobromide (MW >300000), sodium poly-L-glutamate (MW 50000-100000), chitosan, sodium heparinate, pentosan polysulfate, dextran sulfate (MW 8000), DNase I, trypsin, 4-aminobenzoic acid, gentamycin, and MEM non-essential amino acids for cell culturing were from Sigma-Aldrich (St. Louis, MO). DMEM medium, RPMI 1640 medium, and penicillin/streptomycin solution were from Invitrogen. Fetal bovine serum (FBS) was from Atlanta Biologicals (Lawrenceville, GA). Collagenase D and DOTAP transfection reagent were from Roche Applied Science (Mannheim, Germany). 4,6-Diamidino-2-phenylindole (DAPI) was from Molecular Probe (Eugene, OR). HPLC purified hemin hydrochloride (>98% purity) was from Fluka Chemie GmbH (Buchs, Switzerland). Human O-positive blood and O-positive plasma were from The Blood Bank, Hershey Medical Center Hospital, Hershey, PA.

The following antibodies and their isotype controls were from eBioscience (San Diego, CA): anti-mouse CD16/32 antibody (clone 93), FITC-conjugated antibodies against mouse CD11c (clone 418N), NK1.1 (clone PK136), pan-NK cell (clone DX5) and γδ TCR (clone eBioGl3), PE-Cy5-conjugated antibodies against mouse CD11b (clone M1/70), CD80 (clone 16-10A1) and CD86 (clone GL1), PE-Cy5-conjugated hamster IgG and rat IgG2a isotype, allophycocyanin (APC)-conjugated antibodies against mouse CD11c (clone 418N) and CD40 (clone 1C10), and APC-conjugated rat IgG2a isotype. FITC-conjugated anti-mouse TNF-α antibody (clone MP6-XT22), and PE-conjugated antibodies against mouse CD11b (clone M1/70), and IL-12p40/p70 (clone C15.6) were from BD Biosciences (San Jose, CA). Recombinant mouse and human IFN-α were from PBL Biomedical Laboratories (Piscataway, NJ). Duoset ELISA kits for measuring mouse TNF-α, IL-12p40 and IFN-γ, and ELISA kit for human TNF- α, IFN-α measurement, and recombinant mouse FLT3 ligand were from R & D Systems (Minneapolis, MN). ELISA kit for the analysis of human IL-12 and recombinant mouse IFN-γ were from PeproTech (Rocky Hill, NJ). Anti-mouse CD11c antibody-conjugated microbeads, mouse pDC isolation kit II, mouse TCR $\gamma\delta^+$ T cell isolation kit, mouse NK cell isolation kit, human blood DC isolation kit II, FITC-conjugated anti-human CD1c, PE-labeled anti-human CD303, and MS and LS columns for magnetic separation of cells were from Miltenyi Biotec Inc. (Auburn, CA). Rabbit antiserum against *P. falciparum* food vacuole specific P-glycoprotein homologue (Pgh) is a generous gift from Dr. Allen Cowman, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia (23). Alexa Fluor 488-conjugated goat anti-mouse IgG (H + L) and Alexa Fluor 568-conjugated goat anti-rabbit IgG (H + L) were from Molecular Probes.

The Institutional Review Board and the Institutional Animal Care and Use Committee of the Pennsylvania State University College of Medicine, Hershey, respectively, have reviewed and approved the use of human samples and animals for studies reported in this paper.

Mice

The $TLR2^{-/-}$, $TLR4^{-/-}$, $TLR9^{-/-}$ and $MyD88^{-/-}$ mice (all in C57BL/6J background) were provided by Dr. Shizuo Akira, Research Institute for Microbial Diseases, Osaka University, Japan. The C57BL/6J wild type mice were from Jackson Laboratories. All animals were

maintained in a pathogen-free environment, and the animal care was in accordance with the institutional guidelines of the Pennsylvania State University College of Medicine.

Parasite culturing

P. falciparum parasites (3D7 strain) were cultured using O-positive human erythrocytes in RPMI 1640 medium containing 10–20% human O-positive plasma and 50 μg/ml gentamycin under 90% nitrogen, 5% oxygen and 5% carbon dioxide atmosphere as described previously (24). The culture was regularly synchronized by the lysis of the trophozoite and schizont stage parasites using 5% sorbitol (25). The culture was tested every 10-15 days for mycoplasma using Myco Sensor PCR Assay kit from Stratagene (La Jolla, CA).

Isolation and purification of P. falciparum merozoites and food vacuoles

The synchronized cultures of parasite (15-20% parasitemia) at the schizont stage were diluted to 0.1-0.2% hematocrit and incubated. Soon after the majority of the schizont were burst, the culture were harvested and centrifuged at 250 g for 5 min to pellet red blood cells (RBCs) and infected RBCs (IRBCs). The culture supernatants were further centrifuged at 2500 g for 15 min to pellet MZs and FVs. The pellets were suspended in the incomplete RPMI 1640 culture medium, loaded on the top of step-wise cushions of 30%, 45% and 60% percoll solution, and centrifuged at 2500 g for 15 min at 4 °C. The layers on the top of 30% percoll (predominantly MZs) and 45% percoll (FV and MZs) were collected, washed with incomplete medium, suspended in PBS containing 1% BSA (100 μl pellet in 20 ml) and passed through LS columns placed under magnetic field in a MACS separator. Under the magnetic field, FVs containing HZ-iron complex were bound to the columns and MZs were passed through the columns. The columns were washed with PBS containing 1% BSA and then removed from MACS separator. The FVs bound to the columns were eluted with the PBS and collected by centrifugation. The MZs in the column effluents and washes were collected by centrifugation at 2500 g. Thin smears of MZs and FVs were prepared on glass slides, stained with Giemsa, the number of MZs and FVs were counted under light microscope in several fields. On average 1-2 FVs per 100 MZs in MZ preparation, and 1-2 MZs per 100 FVs in FV preparation were observed. Thus, the purity of the MZs and FVs preparations were estimated to be >98%. The identity of the MZs and FVs was confirmed by immunofluorescence analysis. For stimulation experiments, the MZ, FV or IRBC amounts were determined by diluting aliquots of preparations with trypan blue solution and counting under light microscope using hemocytometer. Note: It is important that the parasite culture is tightly synchronized so that the majority of the schizonts are burst within 2-3 h duration and culture is harvested immediately after invasion to avoid MZ lysis. If MZs are lysed, a significant amount of the released DNA-protein complex sticks to the FV membranes.

Isolation of parasite membrane fragments and cytoplasmic material released during the schizont burst

After recovering MZs and FVs as above, the parasite culture supernatants were further centrifuged at 25000 g. The white erythrocyte membranes pelleted on the top of the dark fiber-like parasite membranes were aspirated and the parasite membranes collected. To obtain the cytoplasmic contents of schizonts, the growth medium of synchronous cultures of *P. falciparum* at the schizont stage (200 μl of IRBCs enriched on 65% percoll cushions) was replaced with 5 ml of medium containing 0.5% human plasma. Soon after the majority of schizonts are ruptured, the cultures were harvested, the supernatants were processed to remove residual RBCs and IRBCs, MZs, FVs, and parasite membrane fragments by centrifugation as above. The clear supernatants were considered as the parasite cytoplasmic material dissolved in culture medium and was used for stimulation of cells.

Immunofluorescence analysis of merozoites and food vacuoles

Thin smear of MZs and FVs on glass slides were air dried and fixed with 1% paraformaldehyde in PBS, pH 7.2, for 10 min at room temperature. The slides were rinsed with 10 mM Tris-HCl, 120 mM KCl, 20 mM NaCl and 1 mM EDTA, pH 8.0, containing 1% Triton X-100, incubated with the same buffer for 15 min at room temperature, washed twice with PBS, and blocked with 3% BSA in PBS. The slides were then incubated with 1:400 diluted anti-MSP1 monoclonal antibody and 1:250 diluted rabbit anti-Pgh antiserum followed by 1:400 diluted Alexa Fluor 488-conjugated goat anti-mouse IgG and 1:400 diluted Alexa Fluor 568-conjugated goat anti-rabbit IgG. After washing, the slides were stained with DAPI (0.1 μg/ml) in PBS and examined by fluorescent microscopy using a Nikon Eclipse E1000 microscope and 100× plan apo lens under oil at room temperature and Hamamatsu Orca-ER digital camera. Image capturing and analysis was performed using Image-Pro MC6.0 software.

Treatment of merozoites with trypsin and DNase

The purified MZs suspended in PBS were incubated with 100 μg/ml trypsin at 37 °C for 30 min. The remaining trypsin activity was inactivated by the addition of FBS. For control stimulations, MZs were incubated at 37 °C for 1 h. The MZs were lysed by freeze-thawing and incubated with 100 units/ml DNase at 37 °C for 1 h. The residual enzyme was inactivated by adding EDTA to a final concentration of 2.5 mM and heating at 65 °C for 10 min.

Isolation of parasite genomic DNA

P. falciparum culture (~30% parasitemia) at the trophozoite stage was harvested and parasites from IRBCs were released by treatment with 0.05% saponin in cold PBS. The released parasites were pelleted at 2500 g for 20 min at 4 °C, washed with cold PBS, and suspended in 10 mM Tris-HCl, 20 mM EDTA, pH 8.0, containing 0.5% SDS and proteinase K (25 μg/ml), incubated at 56 °C overnight. After diluting with 2 volumes of water, the solution was extracted three times alternatively with phenol and chloroform, and treated with RNase. The DNA was precipitated with NaOAc and ethanol, washed with 70% ethanol, concentration estimated by measuring absorption at 260 nm, and stored at -20 °C.

Human DNA was isolated from the cultured HEK cells as outlined above for parasite DNA. Mouse DNA was isolated from mouse total spleen cells.

Preparation of parasite and synthetic hemozoin

The FVs, purified as above, were treated with PBS, pH 7.2, containing 0.5% of Triton X-100 and vertexed vigorously to disrupt the membranes. The released HZ was washed 4 times with PBS. Thin smears on glass slides were prepared, stained with Giemsa, and examined under light microscope. The HZ pellet was suspended in complete medium and used for cell stimulation.

Synthetic hemozoin was prepared as reported previously (26). Briefly, hemin chloride (25 mg) was solubilized in 2.5 ml of 0.1 N NaOH and neutralized with 250 μl of 1 N HCl. To the solution was added 10.2 ml of 1 M sodium acetate, pH 5.0, and the suspension was stirred for 2 h at 60 °C. The hemozoin formed was pelleted by centrifugation 14000 g, washed with water, and dried at 37 °C overnight.

Preparation of FLT3 ligand containing conditioned medium

B16 cells expressing retrovirus-coded FLT3 ligand, a generous gift from Dr. Glenn Dranoff, Dana-Farber Cancer Institute of Boston, MA, were cultured in DMEM medium supplemented with 10% FBS, 1% penicillin-streptomycin, non-essential amino acids, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol in roller flasks at 37 °C for 4-5 days. The cell culture supernatant

was used as a source of FLT3 ligand for the differentiation of mouse bone marrow cells into DCs.

Preparation of FL-DCs

FLT3 ligand-differentiated DCs (FL-DCs) were prepared by culturing mouse bone marrow cells in 6-well plates with DMEM complete medium containing 15% of FLT3 ligand medium for 7 or 8 days (27). Alternatively, bone marrow cells were cultured in complete DMEM containing 100 ng/ml purified FLT3 ligand. DCs obtained from both procedures were >90% $CD11c^+$, consisting of ~50% mDCs ($CD11b^+$) and ~50% pDCs ($CD11b^-$). The FL-DCs obtained by both procedures produced comparable levels of TNF-α and IL-12 when stimulated with LPS, Pam_3CSK_4, CpG ODN or MZs.

Isolation of mouse spleen cells

Mouse spleens were homogenized and centrifuged at 200 g. The pellets were suspended in ammonium chloride containing RBC lysis solution, diluted with incomplete DMEM medium, and passed through 70-μm strainer to obtain a single cell suspension. The NK and γδ T cells were isolated by magnetic sorting using the respective cell isolation kit. The purity of the cells was >50%. The NK cells were further purified by FACS sorting using Beckman Cytomation MoFlo High Performance cell sorter after staining with anti-mouse Pan-NK cell antibody; purity of cells was >98%. For isolation of DCs, collagenase D (1 mg/ml in incomplete DMEM medium) was injected into mouse spleens, and incubated at 37 °C for 30 min, then the tissue was crushed and a single cell suspension prepared as above. DCs were isolated by magnetic cell sorting by using anti-mouse CD11c-conjugated microbeads; the purity of cells was ~90%.

Isolation of human DCs, and pDC and mDC subsets

PBMCs were isolated from human blood buffy coat by centrifugation on ISOLYMPH (CTL Scientific Supply Corp., NY) cushions. DCs were enriched from PBMCs by magnetic cell sorting using human blood DC isolation kit II (Miltenyi Biotec Inc.). mDC and pDC subsets were purified by Beckman Coulter Cytomation MoFlo High Performance cells sorter after staining of total DCs with anti-human CD1c and anti-human CD303 monoclonal antibodies. The purity of either cell population was >98%.

Cell stimulation

For analysis of cytokine production, DCs (1×10^5/well) were plated in 96-well flat-bottom plates in duplicate wells and stimulated with MZs and other parasite components or standard TLR ligands (CPG ODN, PAM_3CSK_4 or LPS) for 24 h using 200 μl of culture medium per well. Unless otherwise indicated, 200 μl of MZs, FVs, or IRBCs suspension in culture medium at the amounts indicated in figure legends were used for stimulation of DCs. The culture supernatants were collected and cytokine levels measured by ELISA as reported previously (28). In co-culturing experiments, DCs (1×10^5 cells/well) and NK cells (0.5×10^5/well) or γδ T cells (0.5×10^5 /well) were cultured in 96-well plates using 200 μl of culture medium per well. After 36 h, the culture supernatants were harvested and analyzed for cytokines by ELISA. Wells containing either DCs or NK or γδ T cells alone were used as controls. Transwell experiments were performed by culturing FL-DCs (1×10^6/well in 24-well plates using 1 ml of culture medium) in the lower chamber and NK cells (4×10^5/well) in the upper chamber separated by 0.4 μm membrane (Millipore, Billerica, MA), and both DCs and NK cells were stimulated with MZs (3×10^6/ml). FL-DCs (1×10^6 cells/well) and NK cells (4×10^5 cells/well) cocultured were used as controls. After 36 h, culture supernatants were collected and cytokine levels measured by ELISA.

Analysis of cells for costimulatory molecule and cytokine expression by flow cytometry

DCs (1×10^6) were seeded in 24-well plates, cultured with 1 ml of DMEM complete medium and stimulated with parasite components. After 24 h, cells were collected, stained with dye-conjugated antibodies against costimulatory molecules. For analysis of cytokine expression, DCs were stimulated as above, and 6 h later, Golgi Plug (BD Biosciences) was added. After 5 h, cells were incubated with PE-Cy5-conjugated anti-mouse CD11b antibody and APC-labeled anti-mouse CD11c antibody. After washing, cells were fixed with 2 % formaldehyde for 20 min, permeabilized with 0.5% saponin in PBS containing 1% BSA at room temperature for 10 min, and stained with FITC- or PE-conjugated anti-cytokine antibodies for 30 min. After washing, cells were analyzed by using Becton-Dickinson FACSCalibur and the results were analyzed with CellQuest software (BD Biosciences).

Results

Merozoites are the innate immune stimulatory components of malarial schizont rupture

The systemic illnesses of malaria infection is associated with elevated levels of proinflammatory cytokines produced in response to parasite components released during the schizont burst. To identify the component(s) of the blood stage malaria parasites involved in innate immune responses, we purified various components from the *P. falciparum* schizont burst. The MZs and FVs released to the culture supernatants were isolated by centrifugation on percoll cushions followed by fractionation on magnetic columns. The MZ and FV preparations thus obtained were >98% pure as determined by counting of Giemsa-stained thin smears on glass slides under light microscope. Parasite membrane fragments were isolated by further centrifugation of the culture supernatants. The MZs stained with Giemsa, whereas the FVs did not stain but exhibited the presence of characteristic HZ crystals (Fig. 1). The parasite membrane fragments appeared as dark fibrous material and were devoid of MZs and FVs. The identity and purity of the MZs and FVs were further confirmed by immunofluorescence analysis using anti-MSP1 monoclonal antibody (stains both MZs and FVs), DAPI (specific to MZs), and anti-P-glycoprotein homologue (Pgh) antibody (specific to FV membrane).

The purified *P. falciparum* components were tested for their ability to activate FL-DCs. The MZs efficiently induced the production of TNF-α and IL-12 in a dose-dependent manner (Fig. 2A and B). Similar to MZs, the schizont stage parasite-infected red blood cells (IRBCs) could stimulate FL-DCs robustly in a dose-dependent manner to produce TNF-α and IL-12 (Fig. 3). In contrast, FVs, parasite membrane fragments, and cytoplasmic materials were unable to stimulate cytokine production in FL-DCs (Fig. 2). The MZs could also activate DCs isolated from mouse spleen to produce substantial levels of TNF-α and IL-12 (Fig. 2C and D). Furthermore, MZs induced the maturation of FL-DCs as indicated by the increased surface expression of co-stimulatory molecules, CD40, CD80, and CD86 (Fig. 2E). The schizont ruptured parasite membrane fragments were unable to upregulate the surface expression of CD40, CD80 and CD86, whereas the cytoplasmic materials (parasite culture supernatant) upregulated the expression of CD80 and CD86 at marginal levels; CD40 surface expression remained unchanged (Fig. 2F). The low level of activity observed by the parasite cytoplasmic materials could be due to the presence of trace amounts of DNA derived from the lysis of some merozoites. Together these results demonstrated that MZs are the exclusive component of the schizont rupture that can trigger the activation of DCs.

Activation of DCs by P. falciparum merozoites or IRBCs is TLR9 dependent

TLRs play important roles in the recognition of pathogenic components (15-19). To determine the receptor recognition specificity of MZs and IRBCs, we prepared DCs from mice deficient in TLRs or MyD88 adaptor protein. The MZs or IRBCs were unable to activate DCs deficient in TLR9 or MyD88 and induced the production of TNF-α and IL-12, whereas DCs lacking

TLR2 and TLR4 produced the cytokines at levels similar to that by wild type (WT) DCs (Fig. 4A and B). Further, WT DCs, and TLR2$^{-/-}$ and TLR4$^{-/-}$ DCs stimulated with MZs matured and expressed enhanced levels of co-stimulatory molecules, CD40, CD80 and CD86, whereas DCs deficient in TLR9 or MyD88 were unable to undergo maturation and expression of costimulatory molecules remained unchanged in response to MZ stimulation (Fig. 4C, and Fig. S1). These data indicated that activation of DCs by *P. falciparum* is exclusively dependent on TLR9.

Both proteins and DNA are essential for the activation of DCs by merozoites

The stimulatory activity was unchanged when MZs were lysed by either freeze thawing or sonication. The activity of the lysed MZs or IRBCs was completely abolished when treated with DNase (Fig. 5, and Fig. S2), indicating that DNA is the active component of MZs. However, the purified parasite DNA alone was unable to stimulate DCs. The addition of purified parasite DNA to the DNase-treated MZ lysate restored the activity (Fig. 5). In contrast to parasite DNA, the addition of human or mouse genomic DNA to the DNase-treated MZ lysates showed no activity (Fig. S3), confirming that the parasite DNA is specifically recognized by DCs. Interestingly, treatment with trypsin completely abolished the stimulatory activity of MZs or IRBCs (Fig. 5, and see Fig. S2). When trypsin-treated MZs were combined with DNase-treated MZs, the activity was fully restored. The activity of MZs was lost when heated in boiling water bath for about 5 min. Together these results show that the presence of proteins is essential for the activity of *P. falciparum* DNA.

The requirement of proteins for DNA activity is not restricted to parasite proteins

To determine whether the observed activity is intrinsic to parasite proteins, we examined if exogenous proteins can confer stimulatory activity to parasite DNA. Thus, we tested three distinct types of proteins, namely BSA (a neutral protein), polylysine (PLL, a polycationic protein), and polyglutamic acid (PLG, a polyanionic protein) for their ability to restore the loss of activity when MZs were treated with trypsin. Surprisingly, PLL restored as much as 70-80% of the activity compared to the activity of untreated MZs (Fig. 5). BSA and PLG on the other hand were unable to restore the activity.

The PLL-imparted activity of the purified parasite genomic DNA was also TLR9-dependent (Fig. S4). The PLL-parasite DNA activity was dose dependent with respect to both PLL (up to 2.5 μg/ml) and DNA (Fig. 6A-D). Interestingly, chitosan, a carbohydrate polycationic molecule, also conferred a significant activity to parasite DNA (Fig. 6E and F). In contrast, polyanionic carbohydrates such as heparin, dextran sulfate (DS) and pentosan polysulfate (PS) were unable to impart activity to parasite DNA (Fig. 6E and F, and Fig. S5).

In general, the mammalian cell surfaces carry high levels of net negative charge due to surface display of sialic acids, and heparan sulfate and chondroitin sulfate proteoglycans. Therefore, it appears that DCs are unable to take up the highly negatively charged DNA molecules, which exist as extended structures, because of high degree of charge repulsion. Collectively, the above results indicate that a complex formation involving polycationic proteins and polyanionic DNA is involved in the TLR9-dependent activity of parasite DNA.

The inactivity of purified parasite DNA is due to its inability to enter DCs

We labeled DNA and MZ with DAPI and tested their entry into cells. While a significant amount of MZs was taken up by DCs, the cells were unable to take up the purified DNA (Fig. 7). Therefore, the role of proteins in imparting activity to DNA mainly lies in the entry of DNA to the endosomal compartments of DCs, as reported recently for LL37-DNA complex in skin psoriasis (29). In contrast to parasite DNA, PLL could not impart stimulatory activity to human

or mouse DNA (see Fig. S2), suggesting that gaining entry into cells alone is not sufficient for DNA to activate DCs and distinct elements of parasite DNA appears to be targeted by DCs.

Hemozoin can neither stimulate DCs nor confer activity to parasite DNA

Since the stimulatory activity of IRBCs was completely lost upon treatment with trypsin, despite containing hemozoin and DNA (see Fig. S2), the data suggest that HZ has no role in the activation of DCs. To confirm this, we isolated hemozoin crystals by removing the membranes of the purified FVs by lysing with Triton X-100-containing buffer. The HZ showed no cytokine-inducing activity in DCs (see Fig. 6E and F, and Fig. S5). In contrast to the previous observations that HZ is stimulatory or is able to enhance the activity of parasite DNA (21, 22), both parasite and synthetic HZ were unable to impart stimulatory activity to DNA at various concentrations tested (see Fig. 5E and F, and Fig. S5). These data show that hemozoin is neither a TLR9 ligand nor has ability to present parasite DNA to TLR9. The addition of hemozoin also had no effect (either stimulatory or inhibitory) on the activation of DCs by MZs (Fig. S6).

In the previous study, which reported that hemozoin markedly enhances parasite DNA activity (22), a mixture of uninfected and infected RBCs were passed through magnetic columns and the bound fraction was considered as hemozoin. However, we found that hemozoin-containing matured IRBCs can strongly bind to the magnetic columns. In fact, this procedure is used generally for the isolation of matured IRBCs for synchronized culture of parasites (30). Therefore, the reported activity was due to the presence of parasite protein-DNA complexes in the lysates of IRBCs in the preparation.

mDC and pDC populations of FL- DCs respond differentially to P. falciparum merozoites

Mouse FL-DCs consist of two major subpopulations, $CD11c^{+}CD11b^{+}$ mDCs and $CD11c^{+}CD11b^{-}$ pDCs, in approximately equal proportion. To determine the relative contributions of mDC and pDC populations of FL-DCs to cytokine production in response to MZs, we purified the cells by FACS after staining with anti-CD11c and anti-CD11b monoclonal antibodies. Upon stimulation with MZs, mDCs but not pDCs efficiently produced TNF-α and IL-12 (Fig. 8, A and B). To confirm this observation, the DCs were stimulated with MZs and analyzed for TNF-α and IL-12 expression by intracellular staining using anti-TNF-α and anti-IL-12 monoclonal antibodies. Consistent with the results of ELISA analysis, predominantly mDC population was positive to anti-TNF-α and anti-IL-12 antibodies (Fig. 8C). However, both mDC and pDC populations matured to up-regulate the expression of co-stimulatory molecules when stimulated with MZs, (Fig. 8D).

Human pDCs have been reported to produce IFN-α in response to CpG ODN, LL37-human DNA complex and viral DNA (29,31). We also tested MZs and parasite DNA-protein complex for the activation of DCs obtained from human blood; total DCs and FACS-sorted pDC and mDC populations were analyzed separately. Both unsorted total DCs and sorted pDC population efficiently produced IFN-α in response to stimulation with MZs, PLL-DNA or CpG control (Fig. 9A). Further, unsorted human DCs also produced TNF-α and IL-12 in response to all stimuli (Fig. 9B), whereas sorted pDCs produced TNF-α but not IL-12 (Fig. 9C). As shown in a recent study (32), it is likely that IL-12 is produced mainly by mDCs. However, sorted mDCs were unable to produce IL-12, TNF-α or IFN-α when stimulated with MZs, PLL-DNA or CpG control, but produced TNF-α and IL-12 when stimulated with LPS (Fig. 9A-C). These results are consistent with the lack of TLR9 expression in human mDCs (33). Since human mDCs do not express TLR9 and are not activated directly by MZs (33), it is likely that IL-12 production by mDCs was in response to IFN-α produced by MZ-stimulated pDCs. The addition of IFN-α could not activate sorted human mDCs to produce IL-12, whereas the mixture of sorted pDCs and mDCs produced both TNF-α and IL-12 in response to stimulation with

MZs and PLL-DNA (Fig. 9D). These results indicate that IFN-α produced by pDCs alone is not sufficient for the production of IL-12 by mDCs but, besides IFN-α, other factor(s) produced by pDCs and/or cell-cell contact between both cell types are required.

Both mDC and pDC populations of FL-DCs are essential for the production of IFN-γ by NK cells

When stimulated with MZs, FL-DCs effectively induced the production of IFN-γ by both NK cells and γδT cells (Fig. 10). Given that IL-12 is required for IFN-γ induction and that mDC population but not pDC population produced TNF-α and IL-12 in response to MZs, it was interesting to determine whether only mDC subset help NK cells to produce IFN-γ. Pure mDCs and pDCs (~98% pure), obtained by FACS sorting, were co-cultured separately with spleen NK cells. When stimulated with MZs neither DC type individually could activate NK cells to produce IFN-γ. Priming of FL-DCs with IFN-α substantially increased the production of IFN-γ. Further, when FL-DCs and NK cells were cultured in separate compartments of transwells, and both DCs and NK cells were stimulated, the NK cells failed to produce IFN-γ, although the DCs efficiently produced TNF-α and IL-12. These results indicate that contact between the three cell types is essential for cytokine response by NK cells.

IFN-γ response by NK cells stimulated with P. falciparum protein-DNA complex is TLR9-independent

A previous study has reported that the production of IFN-γ by NK cells in response to *P. falciparum* IRBCs is dependent on the expression of MyD88 in these cells (34). This is surprising considering that NK cell stimulation is mediated by activated DCs rather than through the direct recognition of TLR ligands by NK cells. Consistent with the TLR9-dependent activation of DCs by parasite DNA-protein complex, we found that co-culturing of $TLR9^{-/-}$ or $MyD88^{-/-}$ FL-DCs with NK cells from wild type mice was unable to produce IFN-γ in response to MZ stimulation, whereas co-culturing of FL-DCs and NK cells from WT mice produced strong IFN-γ secretion (Fig. 10). Further, co-culturing of FL-DCs from WT mice with NK cells from $TLR9^{-/-}$ and $MyD88^{-/-}$ mice also produced IFN-γ at levels comparable to that produced by coculturing of DCs and NK cells from WT mice. Neither MZs nor IRBCs were able to directly activate NK cells to produce IFN-γ (Fig. 10). Together these results demonstrated that TLR9 and MyD88 in NK cells are not required for the production of IFN-γ in response to DCs stimulated with *P. falciparum* MZs.

Discussion

By purifying the various components of *P. falciparum* schizont rupture to near homogeneity, we dissected the immunostimulatory part of the parasite responsible for innate immune responses by DCs. Notably, the results show that, among the components released from schizonts, MZs are the inducers of innate immune responses in DCs; parasite FVs, HZ, parasite membrane fragments formed during the differentiation of matured trophozoites into MZs, and parasite cytoplasmic material are non-stimulatory. The activation of DCs by malarial MZs occurs exclusively through the engagement of TLR9-MyD88 signaling pathway. Thus, DCs deficient in TLR9 or MyD88 were completely nonresponsive to malarial MZs or whole schizonts, whereas DCs lacking TLR2 or TLR4 were as efficient as wild type DCs in producing cytokine responses and cell maturation. The TLR9-dependent recognition of *P. falciparum* by DCs is consistent with the previous finding that the IL-12-mediated liver injury in *P. berghei*-mouse malaria model and the activation of human and mouse DCs are mediated by TLR9-MyD88 signaling pathway (35). Also, TLR9-mediated recognition of the parasite has recently been shown to be involved in the acquisition of malaria-specific memory B cells (36).

In malaria infected individuals, the synchronous burst of schizonts releases huge amounts of MZs within a short window of the parasite's 48 h life cycle. The MZs are short lived and as such, they must quickly invade erythrocytes for the efficient survival and parasite propagation. However, in reality, a substantial portion of the released MZs cannot attach and invade erythrocytes. Thus, massive amounts of MZs become the target of DCs, leading to DC maturation, cytokine production, and eventually the development of adaptive immunity.

In this study, we conclusively dissected, for the first time, the stimulatory activity of the malaria parasite. Importantly, our results show that protein-DNA complexes are the immunostimulatory component that is responsible for the inflammatory cytokine production by DCs in response to *P. falciparum*. The parasite proteins are essential for stimulatory activity of an otherwise non-stimulatory parasite DNA. Further, notably, the data show that parasite HZ pigment, which is widely believed to be either a potent immunostimulatory molecule by directly interacting with TLR9 or a carrier of parasite DNA for TLR9 recognition to induce pro-inflammatory responses (21,22), is neither stimulatory by itself for activation of DCs nor is able to confer activity to parasite DNA. Several lines of evidence support our conclusions. These include: (i) the efficient activation of DCs by MZs devoid of HZ; (ii) complete loss of activity upon treatment of MZs or schizont stage IRBCs with DNase, despite the latter containing HZ; (iii) gain of activity of DNase-treated MZs upon the addition of parasite DNA; (iv) total loss of activity when MZs or IRBCs were treated with trypsin, (v) gain of full activity when trypsin-treated MZs was combined with DNase-treated MZ lysates; (vi) the inability of parasite or synthetic HZ to confer activity to parasite DNA. Together these data conclusively show that parasite proteins but not HZ plays a critical role in the TLR9-mediated activation of DCs by malarial DNA.

The ability to confer immunostimulatory activity to *P. falciparum* DNA is not restricted to parasite proteins. PLL, a polycationic protein, imparted a potent stimulatory activity to parasite DNA. Chitosan, a nonprotein polycationic molecule, was also able to impart a significant activity to the parasite DNA. In contrast, polyanionic molecules, including PLG, DS, PS, and heparin were unable to impart activity to the parasite DNA. Thus, it is clear that a complex formation by the ionic interaction with polycationic molecules is essential for the TLR9-dependent activity of parasite DNA. Recently, a cationic antimicrobial peptide LL37, produced during human skin psoriasis, has been shown to complex with DNA released by the tissue, thereby was able to convert the non-stimulatory human DNA into a potent immunostimulatory molecule by facilitating the endosomal entry of DNA (29). The activity was highly specific to the amino acid sequence of LL37 as the peptide with a scrambled sequence was unable to impart activity to human DNA. Thus, it appears that, in addition to aiding endosomal entry of DNA, LL37 converts self-DNA into a structure distinctively different from endogenous components for recognition as a foreign molecule for cell activation. This is in contrast to malarial DNA, which does not require specific polycationic molecule as evident by its conversion into an active immunostimulatory molecule by a diverse type of polycationic molecules, including polypeptides and carbohydrates. By being a molecule of pathogenic origin, malarial DNA is recognized as a foreign molecule by human and mouse DCs. Our findings that polycationic proteins and carbohydrate polymers can convert DNA of pathogenic origin into potent immunostimulatory molecule represent novel findings.

The malaria parasite genome has very high (76%) AT content and CpG motifs are minor constituents (22,37). It is unlikely that CpG motifs of the parasite are the main trigger of stimulatory activity in DCs, although they may contribute to a certain extent (22). A recent study found that the AT-rich motifs of DNA, particularly those containing repeats of several T residues that abundantly occur in parasite DNA, activate cells by engaging TLR9 (38). These findings when considered with our observations that human and mouse genomic DNA, which contain high levels of CpG motifs, are unable to exhibit stimulatory activity in the presence of

PLL or malarial MZ proteins, strongly suggest that the high AT content of parasite DNA is distinctively recognized by TLR9.

Our results further demonstrate that while TLR9 and MyD88 are critically required for activation of DCs, these receptors are not required for the NK cells to produce IFN-γ in response to parasite-stimulated DCs. Thus, $TLR9^{-/-}$ and $MyD88^{-/-}$ DCs stimulated with *P. falciparum* MZs/schizont lysates were unable to activate NK cells, whereas WT DCs stimulated with MZs or IRBCs efficiently activated NK cells to produce IFN-γ. A recent study reported that TLR9-dependent stimulation of DCs was required for the activation of regulatory T cells by malaria parasite (39). This process has been suggested to be involved in the malaria parasite immune evasion. Therefore, TLR9-mediated recognition of malaria parasites by DCs is involved in both immune activation and immune suppression by distinct mechanisms. These findings highlight the complexities involved in malaria immunity.

The results of this study also demonstrate that contact-dependent interactions between mDCs, pDCs and NK cells are essential for the efficient production of IFN-γ by NK cells in response to malarial protein-DNA complex. Although mDC population of mouse FL-DCs efficiently produced IL-12, mDCs are inefficient in activating NK cells in co-cultures to secrete IFN-γ in response to MZs. pDC population was unable to activate NK cells to produce IFN-γ completely. NK cells were also unable to produce IFN-γ when DCs were cultured in separate compartments of transwells. In contrast, when both pDCs and mDCs were cocultured with NK cells, efficient production of IFN-γ was observed, demonstrating that interactions between all three cell types is essential for the optimal production of IFN-γ by NK cells.

Previous studies have shown GPIs purified from *P. falciparum* can activate macrophages through mainly TLR2- and to some extent TLR4-mediated recognition, leading to inflammatory responses. In this study, although merozoites and parasite membrane fragments of schizont rupture contain the membrane-bound GPIs, both parasite components unable to exhibit TLR2-dependent activation of DCs. A minimum of 20 ng/ml of purified GPIs are required to observe a detectable level of cytokine responses by macrophages primed with IFN-γ, and saturated level of cytokine response requires at least 200 ng/ml of GPI concentration (40). The dose of merozoites required for the saturated level of cytokine responses contain an estimated amount of ~5 ng/ml of GPIs (41). Thus, it is evident that the potency of TLR9-dependent activity of DNA-protein complex in merozoites is markedly higher than the ability of parasite membrane-bound GPIs to activate DCs and it appears that the contribution of GPIs toward activation of DCs *in vivo* is limited.

Our data have important considerations for the development of an effective vaccine for malaria. Several MZ proteins, including MSP1, MSP2, MSP3, AMA1, and EBA-175 have been extensively studied as vaccine candidates, either as a single protein or as multi-subunit structures (42,43). Although these candidate proteins are in various stages of clinical trials, so far none has been proved to be useful as an efficacious vaccine. Because of low immunogenicity of malarial antigens, adjuvants are essential for efficient immune responses. Several adjuvants, including alum, gels, and oil-based components have been studied but all exhibit various levels of toxicity (44,45). In this regard, MZs or whole IRBCs may prove to be very effective for malaria vaccination as they contain repertoire of antigens involved in MZ invasion of erythrocytes and immune-boosting protein-DNA complex for strong adjuvant activity. Since MZs or IRBCs can effectively stimulate DCs through TLR9 recognition, this process will likely to induce an effective adaptive immunity. Recently, MZs have been proposed for malaria vaccination (46). Our data strongly support this notion. Furthermore, our results provide a conceptual framework for strategies aimed at designing DNA-protein antigen complexes as a malaria vaccine.

In summary, this study clearly demonstrates that protein-DNA complex is the exclusive component that activates DCs and HZ has no role in the DNA-mediated activation of DCs by malaria parasite, clarifying the hitherto widely held belief that HZ is either directly or indirectly immunostimulatory. The results set a stage for refocusing of studies that aimed at understanding of the initiation and regulatory mechanisms of malaria immunity by DCs. More importantly, the involvement of the parasite protein-DNA complex-mediated immune responses may have potential for the development of immune-based therapeutics or a vaccine for malaria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

IRBC	infected red blood cell
MZ	merozoites
FV	food vacuole
HZ	hemozoin
pDNA	parasite DNA
Pgh	P-glycoprotein homologue
PLL	poly L-lysine
PLG	poly L-glutamic acid
DS	dextran sulfate
PS	pentosan polysulfate
FL-DC	FLT3 ligand differentiated DC
mDC	myeloid DC
pDC	plasmacytoid DC

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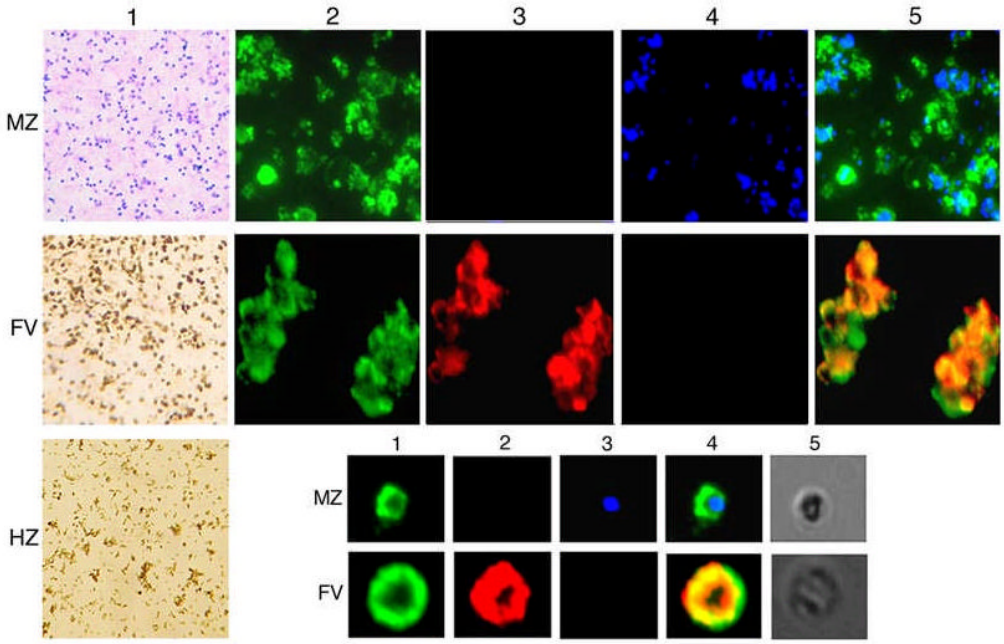

FIGURE 1.

Analysis of *P. falciparum* MZs, FVs, and HZ for purity. (*Panel 1*) Light micrographs of Giemsa-stained MZ, FV and HZ. (*Panels 2-4*) Immunofluorescent micrographs after staining with anti-merozoite surface protein-1 monoclonal antibody (*2*), rabbit anti-Pgh antibodies (*3*) or DAPI (*4*). (*Panel 5*) Merge of micrographs 2, 3 and 4. (*Inset*) Enlarged immunofluorescent micrographs of a single MZ and FV stained as above; phase-contrast micrographs of MZ and FV (*5*).

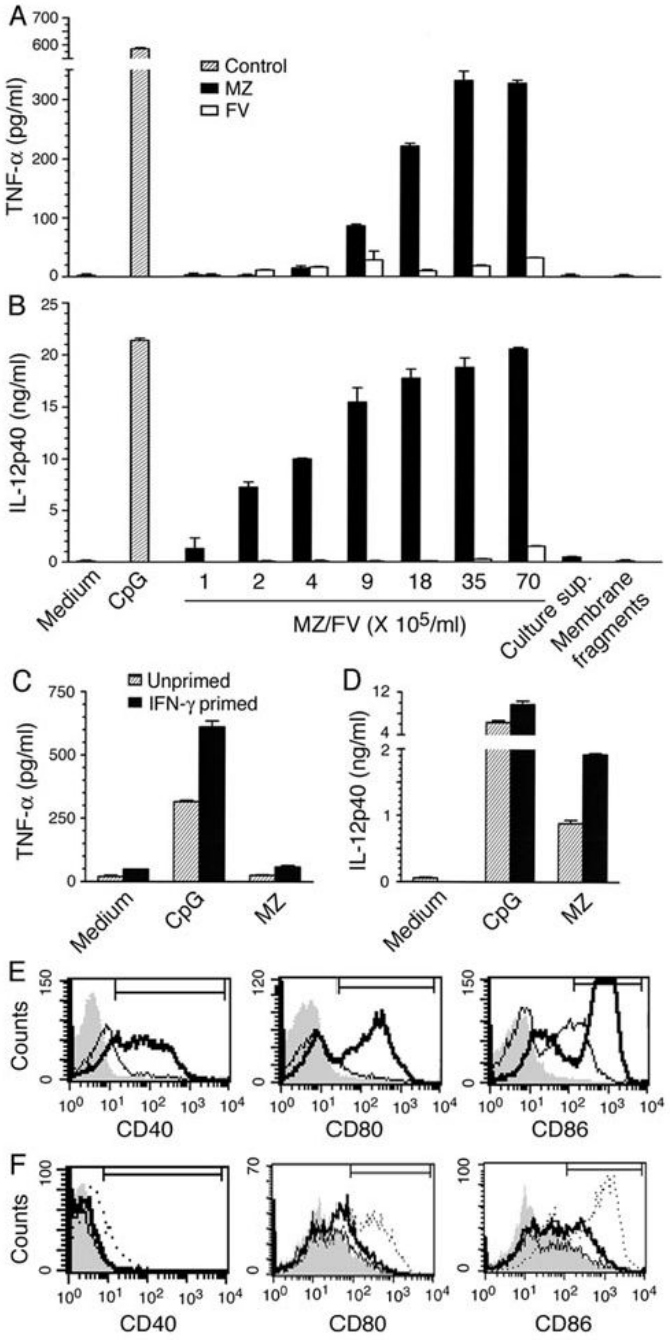

FIGURE 2.

MZs are the exclusive components of *P. falciparum* schizont rupture that induce the production of inflammatory cytokines in DCs. (*A* and *B*) TNF-α and IL-12 produced by FL-DCs (1×10^5 cells/well in 200 μl of culture medium) stimulated with MZs, FVs (each at the indicated doses), parasite membrane fragments (2×10^7 IRBC equivalent/well), or parasite cytoplasmic materials (2×10^7 IRBC equivalent/well). CpG-1826 (2 μg/ml) was used as a control stimulant in all experiments. Cytokines secreted into culture media were measured by ELISA. Data are representative of six independent experiments, each performed in duplicates. In all figures, error bars represent mean values ± SEM. (*C* and *D*), TNF-α and IL-12 produced by unprimed and IFN-γ -primed mouse splenic DCs (1×10^5 cells/well in 200 μl of culture medium)

stimulated with MZs (3×10^6/ml; 6×10^5/well). Data are representative of three independent experiments. (*E and F*) Surface expression of costimulatory molecules (CD40, CD80, and CD86) by FL-DCs stimulated with the MZs (3×10^6/ml/well, *E*), and schizont ruptured membrane fragments and cytoplasmic material (equivalent of 1×10^8 IRBCs in 1 ml of culture medium, *F*) in 24 well plates. (*E*), Shaded area, thin line, and thick line represent, respectively, the IgG isotype controls, unstimulated cells, and cells stimulated with MZs (3×10^6/ml/well). (*F*), Shaded area, thin line, thick line, and dashed line represent, respectively, unstimulated cells, stimulated with membrane fragments, cytoplasmic material and CpG control.

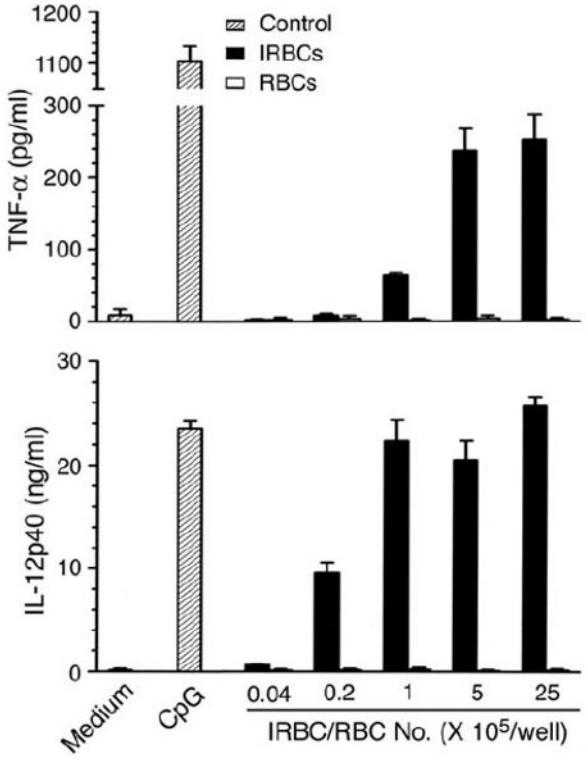

FIGURE 3.

P. falciparum IRBCs activate DCs in a manner similar to that by MZs. ELISA measurement of TNF-α and IL-12 produced by FL-DCs (1×10^5/well in 200 μl of culture medium) stimulated with the indicated doses of the late stage (trophozoites and schizonts) IRBCs. Cells stimulated with RBCs and CpG-1826 (2 μg/ml) were analyzed as controls. Data are representative of four independent experiments.

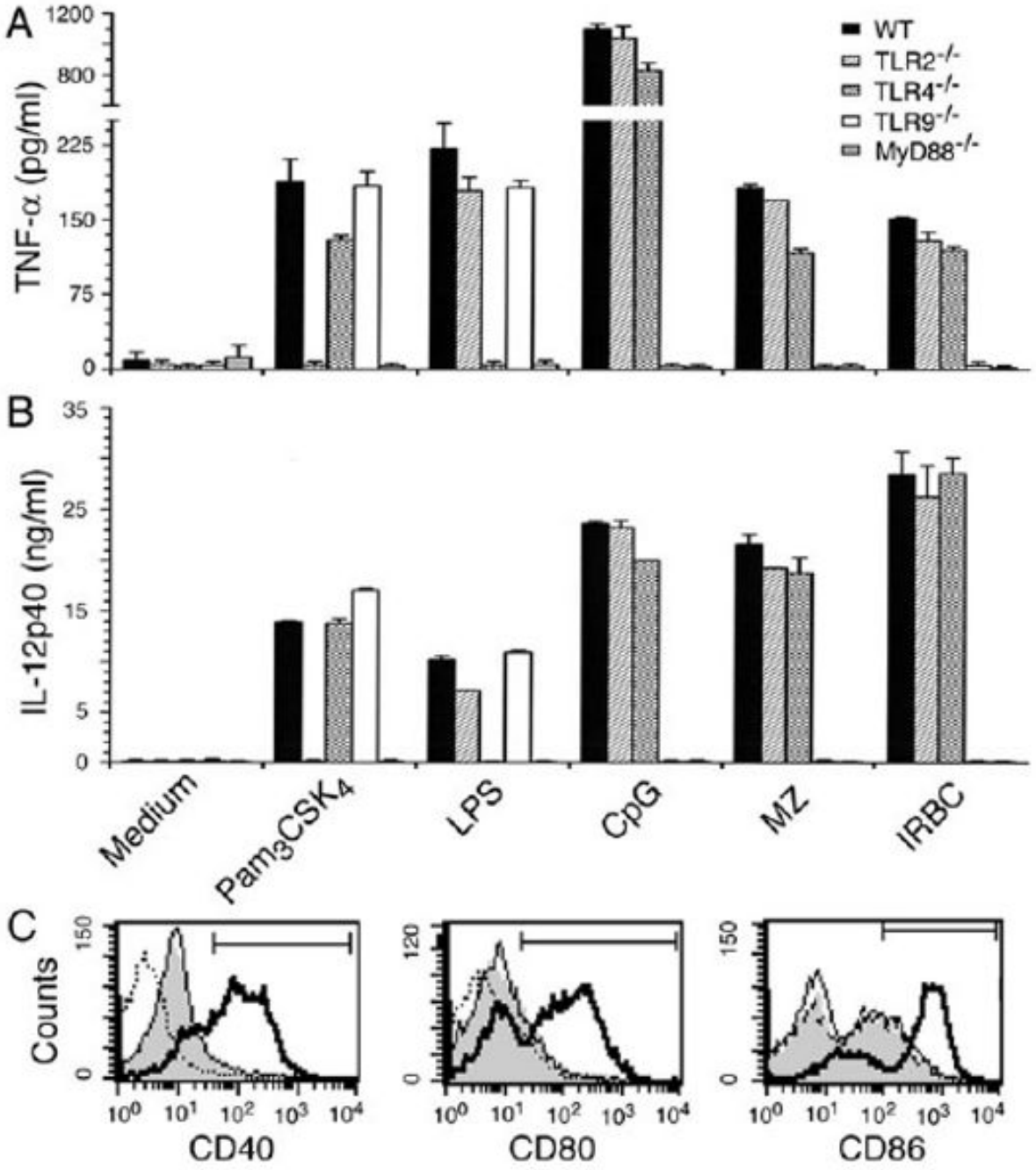

FIGURE 4.

Activation of DCs by MZs and IRBCs is mediated by TLR9. (*A* and *B*) Analysis of TNF-α and IL-12 by WT, and TLR2, TLR4, TLR9 and MyD88 deficient FL-DCs (1×10^5/well in 200 μl of culture medium) stimulated with MZs (3×10^6/ml; 6×10^5/well) or IRBCs (1.5×10^6/ml; 3×10^5/well). Pam_3CSK_4 (TLR2 ligand, 10 ng/ml), LPS (TLR4 ligand, 100 ng/ml), and CpG (TLR9 ligand, 2 μg/ml) were used as control stimulants. Data are representative of six independent experiments. (*C*) Co-stimulatory molecules expressed by WT (thick lines), $TLR9^{-/-}$ (shaded areas) and $MyD88^{-/-}$ (dashed line) FL-DCs stimulated with MZs (3×10^6/ml/well) and unstimulated WT FL-DCs (thin lines).

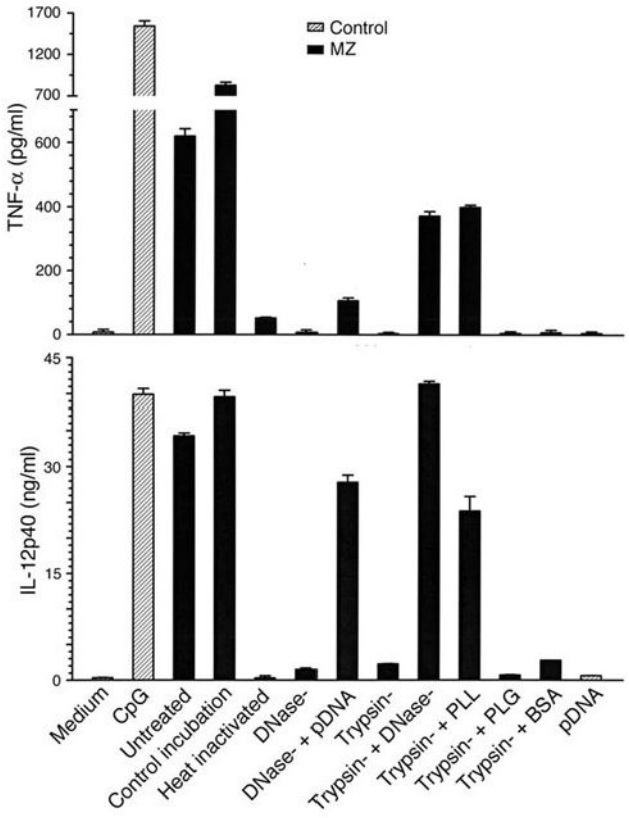

FIGURE 5.

The activation of DCs by *P. falciparum* merozoites involves recognition of a parasite protein-DNA complex. TNF-α and IL-12 produced by FL-DCs (1×10^5/well in 200 μl of culture medium) stimulated with untreated, DNase-treated MZs (DNase-), trypsin-treated MZs (Trypsin-), mixture of DNase- and trypsin-treated MZs. The indicated exogenous proteins were added to trypsin-treated MZs at 2.5 μg/ml; in each case, 3×10^6/ml of MZs (6×10^5/well) were used. Parasite DNA was tested at 8 μg/ml. Data are representative of four independent experiments. DCs stimulated with CpG (2 μg/ml) were analyzed as a control. MZs incubated at 37 °C for 1 h were tested as controls.

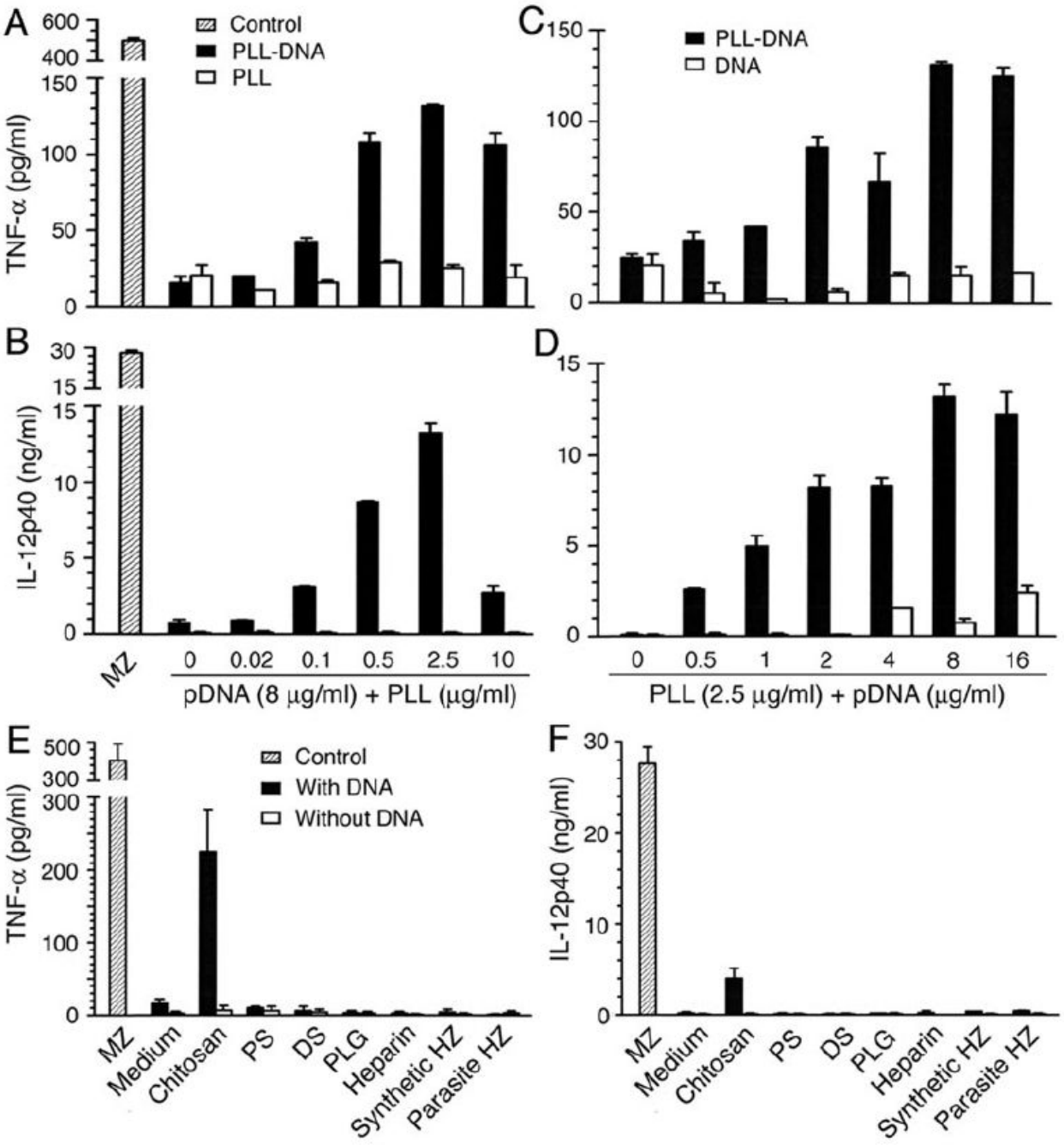

FIGURE 6.

PLL converts *P. falciparum* inactive DNA into a potent stimulatory molecule. (*A* and *B*) TNF-α and IL-12 produced by FL-DCs (1×10^5/well in 200 μl of culture medium) stimulated with 8 μg/ml of parasite DNA containing the indicated concentrations of PLL. (*C* and *D*), TNF-α and IL-12 produced by FL-DCs stimulated with DNA containing in each case 2.5 μg/ml of PLL. (*E* and *F*) TNF-α and IL-12 produced by FL-DCs stimulated with parasite DNA (8 μg/ml) containing PLG, PS, DS, chitosan, heparin, parasite hemozoin or synthetic hemozoin (5 μg/ml each). All these reagents were also tested at different doses (see Fig. S5). Data are representative of five independent experiments. MZs (3×10^6/ml; 6×10^5/well) were analyzed as control stimuli.

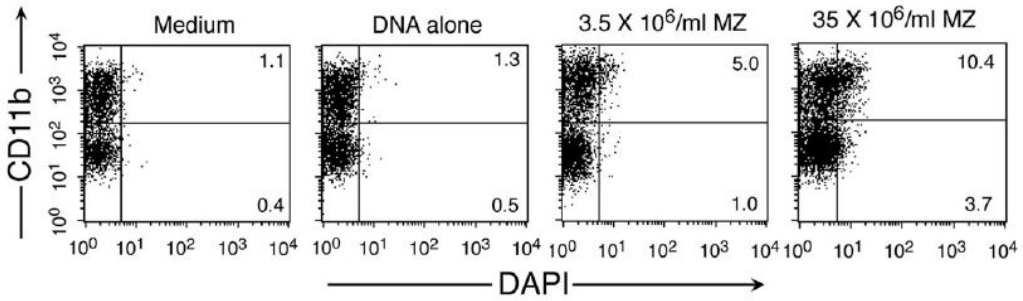

FIGURE 7.

The mDC subset of FL-DCs can uptake *P. falciparum* MZs and DNA complex more efficiently than the pDC subset. Parasite DNA and MZs were stained with DAPI (0.1 μg/ml in PBS) and FL-DCs (1×10^6/well in 24-well plates) were stimulated with stained DNA (8 μg/ml) or MZs (at the indicated amounts) in 1 ml of culture medium. The uptake of parasite DNA and MZs by DCs was measured by flow cytometry. Data are representative of two independent experiments.

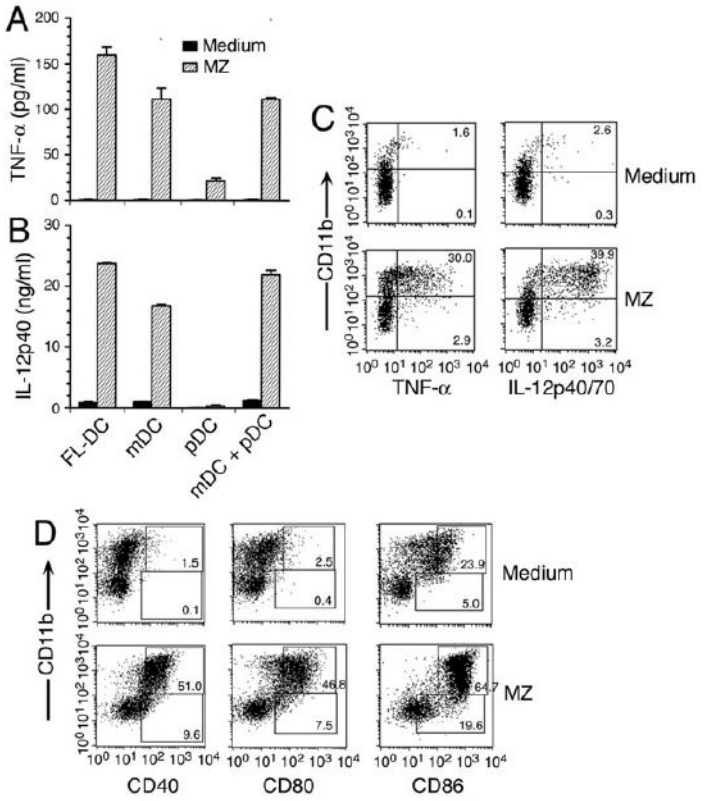

FIGURE 8.

P. falciparum MZs can activate both myeloid DCs and plasmacytoid DCs to express costimulatory molecules, but only former DC population produce TNF-α and IL-12. (*A* and *B*) TNF-α and IL-12 production by sorted mDC and pDC populations (each 5×10^4 cells/well in 200 μl of culture medium) of FL-DCs and by the reconstituted mixture of sorted mDCs and pDCs (mDC + pDC) stimulated with MZs (3×10^6/ml; 6×10^5/well). (*C*) TNF-α and IL-12 produced by FL-DCs (1×10^6/well in 24-well plates) stimulated with MZs (3×10^6/ml/well) were analyzed by intracellular staining. Data shown in each (*A*), (*B*) and (*C*) are representative of three independent experiments. (*D*) Costimulatory molecules expressed by FL-DCs (1×10^6/well in 24-well plates) stimulated with MZs (3×10^6/ml/well). Unstimulated FL-DCs were analyzed as controls. Experiments were done two times.

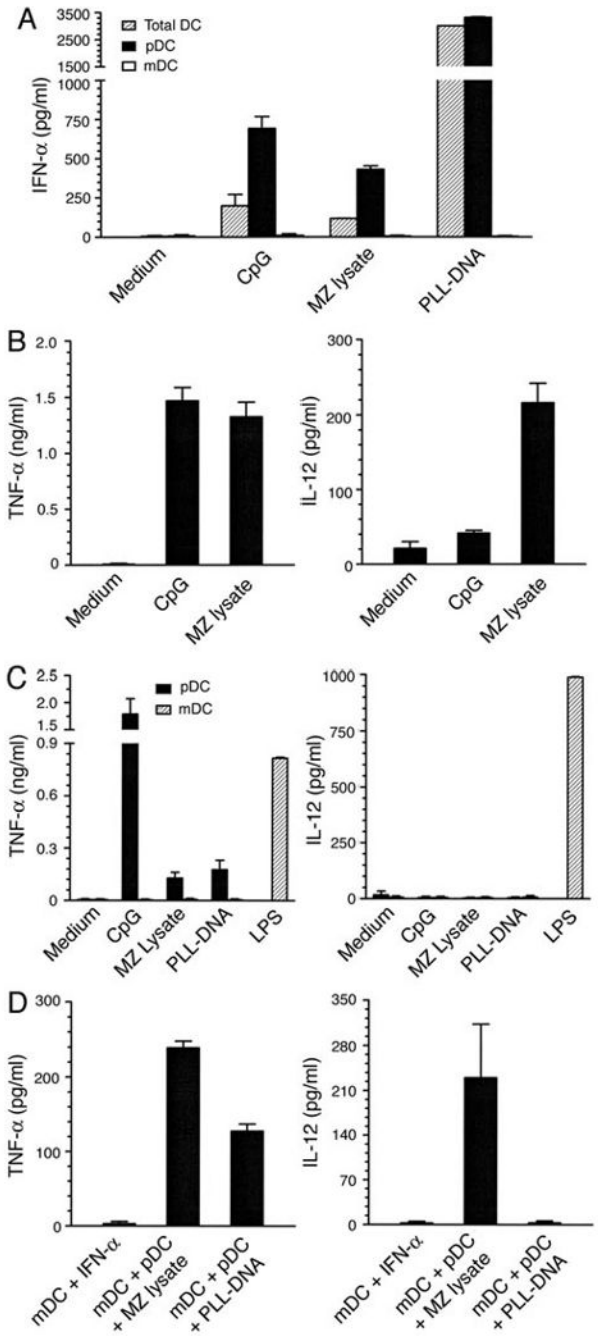

FIGURE 9.

Human DCs efficiently produce IFN-α, TNF-α, and IL-12 in response to malaria parasite. DCs isolated from human blood were sorted by FACS into mDC and pDC. (*A*) Unsorted DCs (1×10^5/well in 200 μl of culture medium) and sorted DCs (each 1×10^5/well in 200 μl of culture medium) were stimulated with CpG-2006 (2 μg/ml), MZ lysates (MZ containing 2 μg DNA/ml) or parasite DNA (8 μg/ml) + PLL (2.5 μg/ml). IFN-α released into the cultured medium was measured by ELISA. (*B*) In separate experiments, human blood total DCs (1×10^5 cells/well in 200 μl of culture medium) were stimulated with CpG-2006 (2 μg/ml) control or MZ lysates (MZ, 5 μg DNA/ml) (*C*) Production of cytokines produced by sorted human pDCs and mDCs stimulated with MZ lysate (equivalent of 2 μg/ml DNA), parasite DNA (8 μg/ml) + PLL

(2.5 μg/ml), and CpG-2006 (2 μg/ml) and LPS (100 ng/ml) controls each in 200 μl culture medium. (*D*) Sorted human mDCs or mixtures of sorted pDCs and mDCs were stimulated with IFN-α (1000 pg/ml), MZ lysate or parasite DNA-PLL as indicated at concentrations outlined in panel *C*. In all cases, TNF-α and IL-12 secreted into culture medium were measured by ELISA.

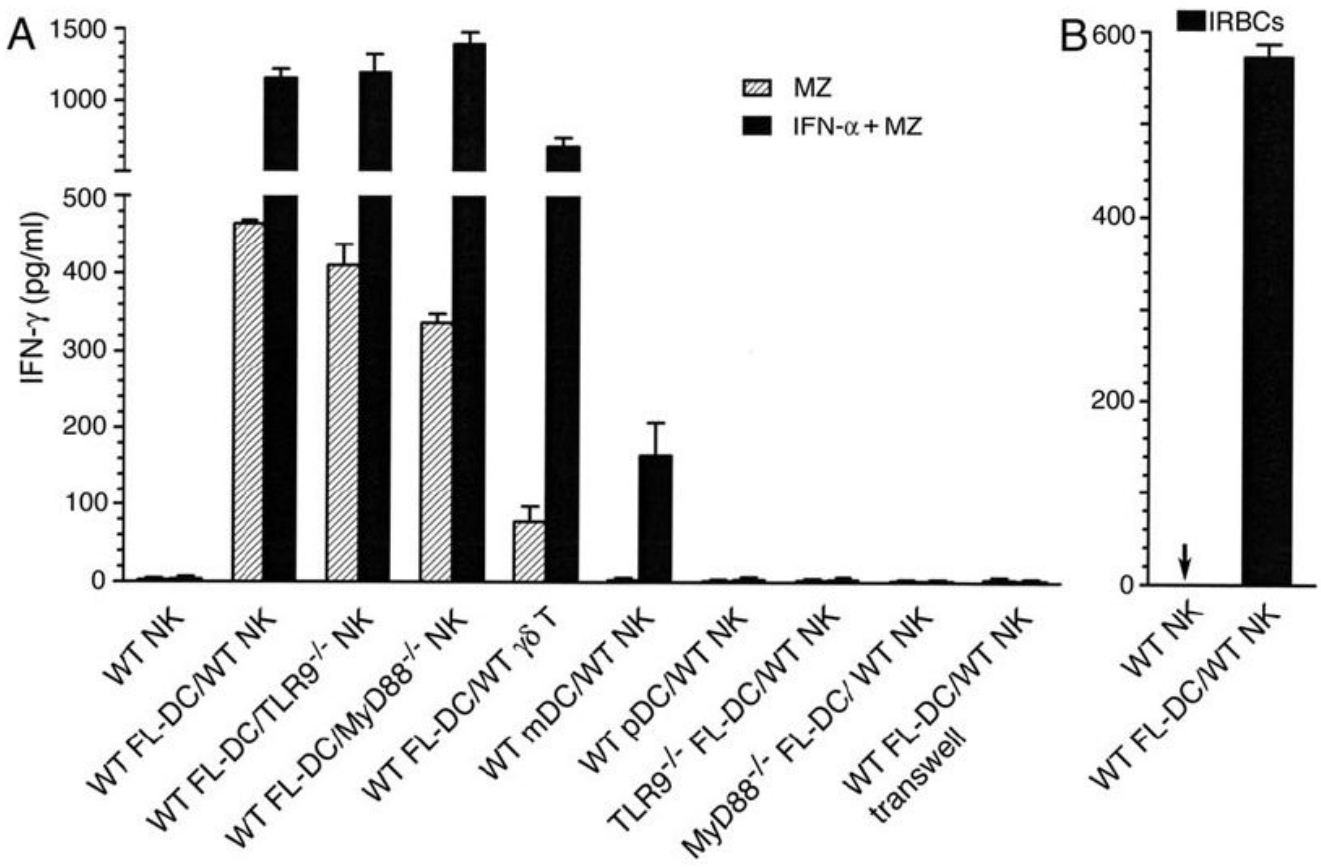

FIGURE 10.

Merozoite- and IRBC-stimulated DCs can activate NK and γδ T cells to produce IFN-γ. (*A*) WT FL-DCs (1×10^5 cell/well) cocultured with NK cells (5×10^4 cells/well) from WT, TLR9$^{-/-}$ or MyD88$^{-/-}$ mice or WT γδ T cells (5×10^4/well) all in 96 well plates using 200 μl of culture medium. In each case, cells were stimulated with MZs (3×10^6/ml; 6×10^5/well) or MZs (3×10^6/ml; 6×10^5/well) plus IFN-α (500 units/ml). Similarly, TLR9$^{-/-}$ or MyD88$^{-/-}$ FL-DCs (1×10^5/well in 200 μl of culture medium) cocultured with WT NK cells (5×10^4/well) were stimulated with MZs (3×10^6/ml; 6×10^5/well) or MZs (3×10^6/ml; 6×10^5/well) plus IFN-α (500 units/ml). Sorted mDC and pDC populations (each 5×10^4/well in 200 μl of culture medium) of WT FL-DCs cocultured with WT NK cells were also stimulated as above. To examine the cell-cell contact requirement for IFN-γ production, FL-DCs (1×10^6/well) and NK cells (4×10^5/well) were cultured in different compartments of 24-well transwells, and both DCs and NK cells were stimulated with MZs (3×10^6/ml/well) or MZs (3×10^6/ml/well) plus IFN-α (500 units/ml). Data are representative of five independent experiments. (*B*) IFN-γ production after stimulation with IRBCs; arrow indicates that IFN-γ was not detectable. Data are representative of three independent experiments.