T-cell activation and cytokine production via a bispecific single-chain antibody fragment targeted to blood-stage malaria parasites

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A novel bispecific single-chain antibody fragment (biscFv) has been constructed to address the possibility of a new approach to malaria therapeutic drug development. The biscFv consists of 2 different single-chain antibody fragments linked by a flexible peptide linker (Gly_4-$Ser)_3$. Of the 2 scFv fragments, one is directed against a conserved epitope of the 19-kDa C-terminal fragment of the major surface protein of human malignant malaria parasite, *Plasmodium falciparum,* and the other is directed against the CD3 antigen of human T cells. The biscFv expressed by a recombinant baculovirus retained the antigen-binding properties of the corresponding univalent single-chain antibody fragments and formed a bridge between *P falciparum* and T cells. In cooperation with T cells, the biscFv specifically induced not only interferon γ and tumor necrosis factor α, but also a significant increase of merozoite phagocytosis and growth inhibition of *P falciparum* in vitro. Thus, the biscFv possesses highly selective malaria-targeting properties and stimulates T cells to induce cytokines, presumably resulting in activation of macrophages, neutrophils, and natural killer cells, and parasite killing in vivo. (Blood. 2003;101:2300-2306)

Introduction

Malaria kills between 1.5 and 2.7 million people each year, and between 300 and 500 million others fall ill from it, often severely.[1] The greatest cause of mobility and mortality is caused by infection with *Plasmodium falciparum.* The emergence and spread of drug resistance to common antimalarial drugs and the current unavailability of vaccines against any form of malaria has increased the urgency of new drug development efforts for the control of *P falciparum* malaria.

Bispecific single-chain antibody fragments (biscFvs) are proteins that have 2 different binding specificities: the first binding site is specific for an antigen present on the target cell, and the second binding site recognizes an antigen on immune effector T cells involved in signal transduction and T-cell activation. It is thought that biscFvs bridge target cells and effector cells, resulting in target-cell destruction. The potential use of biscFv for immunotherapy has been reported in several models. For instance, anti-CD3 biscFvs with antitumor scFv have been shown to activate T cells and mediate redirected cellular cytotoxicity in vitro and in vivo, resulting in tumor lysis.[2-6] Several clinical trials have supported the clinical potential of anti-CD3 biscFvs with antitumor scFv.[7-9] In addition, a biscFv composed of CD4 and anti-CD3 also has been reported to retarget T cells against gp120-expressing HIV-infected cells.[10-12] Thus, application of biscFvs appears to be a promising avenue for malaria immunotherapy because biscFvs do not rely on the induction of antibody production but rather entail the stimulation and targeting of cell-mediated mechanisms. Moreover, unlike antibodies, which are often strain specific, biscFvs would act upon *P falciparum* strains of diverse genetic makeup.

Conserved epitopes expressed on the surface of parasite-infected erythrocytes or the merozoite surface could serve as potential targets for selective immunotherapeutic molecules. PfMSP-1 is a prominent antigen in malaria vaccine development and has been found to induce antibodies that bind to the merozoite surface, inhibit parasite growth in vitro, and induce a protective immune response against malaria infection in nonhuman primates in vivo.[13-16] Although portions of the PfMSP-1 gene undergo allelic variation, 19-kDa C-terminal processing product of PfMSP-1 ($PfMSP\text{-}1_{19}$), which appears to be an important target of the protective immune response, is highly conserved and may serve as a potential target of immunotherapy. Taken together, the bridging between blood-stage parasites and T cells via anti-CD3 biscFv with anti–$PfMSP\text{-}1_{19}$ scFv would be expected to trigger cellular activation and the release of the cytokines responsible for macrophage or neutrophil activation, resulting in phagocytosis and killing of blood-stage malaria parasites. To our knowledge, no biscFv has been exploited to date as an immunotherapeutic drug intervention in the field of developing new drugs for malaria.

In this paper we describe a genetically engineered biscFv consisting of anti-CD3 scFv and anti–$PfMSP\text{-}1_{19}$ scFv. This 5.2-OKT3 biscFv was expressed in insect cells using a recombinant baculovirus expression system. The 5.2-OKT3 biscFv antigen-binding characteristics, in vitro merozoite targeting properties, and in vitro blood-stage parasite killing potential of 5.2-OKT3 biscFv-activated effector cells are presented. The potential usefulness of this biscFv approach for activation of T-cell–mediated antimalarial mechanisms is discussed.

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Materials and methods

Parasites, cell lines, and antibodies

Hybridoma clones 5.2 and OKT3 were obtained from the American Type Culture Collection (Rockville, MD). These 2 hybridoma cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum. The 5.2 and OKT3 monoclonal antibodies (mAbs) were purified from ascites fluid using E-Z Step (Pharmacia Biotech, Uppsala, Sweden). Sf9 cells and High Five cells (Invitrogen, San Diego, CA) were maintained as described previously.[17] Asynchronous cultures of *P falciparum* (FCR3 strain, a strain identical to the Wellcome strain, kindly donated by Dr Kanoh) were maintained in human erythrocytes in complete medium as described previously.[18]

Cloning and sequencing of the *5.2* and *OKT3 V* genes

mRNA was extracted from 1×10^7 5.2 hybridoma cells using FastTrack 2.0 mRNA Isolation Kit (Invitrogen), and first-strand cDNA was synthesized from the mRNA with reverse transcriptase (RT) using the First-Stranded cDNA Synthesis Kit (Novagen, Madison, WI). The cDNA was used as a template for polymerase chain reaction (PCR) amplification of the V_H and V_L genes of 5.2 mAb, using *Taq* 2000 DNA polymerase (Stratagene GmbH, Heidelberg, Germany) and the primer sets in the Mouse Ig-Prime Kit (Novagen). The single-chain genes were assembled by adding a linker $(Gly_4Ser)_3$ and inserted into the phage display vector pCANTAB5E (Pharmacia Biotech) to construct pCANTAB5E-5.2scFv. Binding functionality of the single-chain to $PfMSP\text{-}1_{19}$ was first analyzed by phage display system (Pharmacia Biotech). The selected single-chain genes were sequenced and the nucleotide sequence data have been deposited in GenBank database under the accession numbers AB028875 and AB028876. To construct a baculovirus transfer vector for the *5.2 scFv* gene, the V_H and V_L genes of 5.2 mAb were separately amplified from pCANTAB5E-5.2scFv using cloned *Pfu* DNA polymerase (Stratagene GmbH) with specific sets of primers, $p5.2V_H\text{-}1/p5.2V_H\text{-}2$ and $p5.2V_L\text{-}1/p5.2V_L\text{-}2$, respectively (Table 1). After amplification of the V_H and V_L genes, overlapping PCR was performed to construct the *5.2 scFv* gene under the same conditions, but with the $p5.2V_H\text{-}1$ and $p5.2V_L\text{-}2$ primers. The *5.2 scFv* gene fragment was digested with *Bam*HI and *Sfi*I, and inserted into *Bam*HI/*Sfi*I-digested pBACgus-13.1scFv,[17] which contains the first 21 codons of the melittin gene signal sequence followed by *Bam*HI site. The resulting transfer vector was designated pBACgus-5.2 scFv.

For the cloning of the *OKT3* V_H and V_L genes, the first-stranded cDNA were similarly synthesized from mRNA extracted from OKT3 hybridoma cells. Based on the sequence information of the *OKT3* V_H and V_L genes,[19] the *OKT3* V_H and V_L genes were amplified from the cDNAs with specific sets of primers, $pOKT3V_H\text{-}1/pOKT3V_H\text{-}2$ and $pOKT3V_L\text{-}1/pOKT3V_L\text{-}2$, respectively (Table 1). After amplification of the V_H and V_L genes, overlapping PCR was performed to construct the *OKT3 scFv* gene under the same conditions, but with the $pOKT3V_H\text{-}1$ and $pOKT3V_L\text{-}2$ primers. The *OKT3 scFv* DNA was digested with *Bam*HI and *Sfi*I, and inserted into *Bam*HI/*Sfi*I-digested pBACgus-13.1scFv. The resulting transfer vector was designated pBACgus-OKT3 scFv. Thus, the *5.2 scFv* and *OKT3 scFv* genes are nonhuman genes obtained from mouse hybridoma cells.

For the construction of a biscFv, the *OKT3* gene with $(Gly_4Ser)_3$ linker sequence at the 5′ end was amplified from pBACgus-OKT3 scFv with $pOKT3V_H\text{-GC}$ and $pOKT3V_L\text{-}2$ primers (Table 1). The PCR product was digested with *Bgl*I, and inserted into *Sfi*I-digested pBACgus-5.2 scFv. The resulting transfer vector was designated pBACgus-5.2-OKT3 biscFv.

Expression and purification of scFvs

Recombinant baculoviruses were generated by cotransfection of Sf9 cells with pBACgus-5.2 scFv, pBACgus-OKT3 scFv, or pBACgus-5.2-OKT3 biscFv and linearized baculovirus DNA BacVector-3000 (Novagen) according to the manufacturer's protocol. For protein production, High Five cells were grown to a density of 1.5×10^6 cells/mL in Ex-Cell 405 medium (JRH Bioscience, Lenexa, KS). Cells were infected with recombinant baculovirus at a multiplicity of infection of 3 (MOI = 3) and incubated at 27°C for 3 days. All scFv antibodies were purified from culture supernatants by affinity chromatography on a Ni-NTA Superflow column (Qiagen GmbH, Hilden, Germany) following chromatography on a Q-Sepharose anion exchange column (HiPrep 16/10 QXL, Pharmacia Biotech). The purity of the proteins was checked by silver staining of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and protein yield was estimated by comparison with bovine serum albumin (BSA) standards.

Immunoblotting

Preparation of purified native gp195 and 42-kDa PfMSP-1 C-terminal polypeptide ($rPfMSP\text{-}1_{42}$) expressed by recombinant baculovirus was described previously.[20] The protein samples were separated on SDS-PAGE and transferred to nitrocellulose membrane. Following blocking with BSA, the membranes were incubated with the purified scFvs at a concentration of 50 μg/mL. Bound scFvs were detected with biotinylated mouse anti-FLAG M2 mAb (Eastman Kodak, Rochester, NY) followed by development with 5-bromo-4-chloro-3-indolylphosphate *p*-toluidin salt/nitroblue tetrazolium chloride substrate (Life Technologies, Rockville, MD) as described previously.[17]

Preparation of lymphocytes

Human peripheral blood mononuclear cells (PBMCs) were obtained from heparinized peripheral blood of a single donor with no previous exposure to malaria by centrifugation on Lymphoprep (Nycomed, Oslo, Norway). The cells were washed and resuspended in malaria complete medium. PBMC preparations were used for flow cytometry, intercellular bridge formation, cytokine, growth inhibition, and phagocytosis assays.

Flow cytometry analysis

T cells were isolated from PBMCs by nonadherence to a nylon wool column (WAKO Pure Chemical Industries, Tokyo, Japan) as described previously.[21] Aliquots of 2×10^6 T cells in 200 μL RPMI 1640 were mixed with protein samples or parent mAbs at a final concentration of 50 μg/mL. Binding of scFvs to T cells was determined by flow cytometry as described previously.[19] In all experiments, freshly prepared PBMCs were used without any pretreatment.

Table 1. Oligonucleotide primers used for amplification and construction of scFvs

$p5.2V_H\text{-}1$	5′-GGG*GATCC*GGACTACAAGGACGACGATGACAAGATCCTAGCACAGATCCAATTGGTGCAGTCTGGACCTGAGCTG-3′
$p5.2V_H\text{-}2$	5′-GCCACCGCCAGAGCCACCTCCGCCTGAACCGCCTCCACCTGTCGACGATGAGGAGACTGTGAGAGTGGTGCCTTGGCCCCAGACGG-3′
$p5.2V_L\text{-}1$	5′-GGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGATATCGACATTGTGATGTCACAGTCTCCATCCTCC-3′
$p5.2V_L\text{-}2$	5′-GGAGGA*GGCCCCCTGGGCCC*CTGTTTTATTTCCAGCTTGGTCCCCCCTCCGAACGTGTACAGATTATAAGATTG-3′
$pOKT3V_H\text{-}1$	5′-GGG*GATCC*GGACTACAAGGACGACGATGACAAGATCTCCCAGGTCCAGCTGCAGCAGTCTGGGGCTGAACTGGCAAGA-3′
$pOKT3V_H\text{-}2$	5′-GCCACCGCCAGAGCCACCTCCGCCTGAACCGCCTCCACCTGTCGACGATGAGGAGACTGTGAGAGTGGTGCCTTGGCCCCAGTAG-3′
$pOKT3V_L\text{-}1$	5′-GGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGATATCCAAATTGTTCTCACCCAGTCTCCAGCAATC-3′
$pOKT3V_L\text{-}2$	5′-GGAGGA*GGCCCCCTGGGCCC*CCGGTTTATTTCCAACTTTGTCCCCGAGCCGAACGTGAATGGGTTACTACTCC-3′
$pOKT3V_H\text{-GS}$	5′-*GCCCAGGGGGC*GGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGAAGATCTCCCAGGTCCAGCTGCAGCAGTCTGGG-3′

Sequences encoding $(Gly_4Ser)_3$ are underlined.
The restriction enzyme recognition sites are in italic.

Intercellular bridge formation

$CD8^+$ T cells were isolated from PMBCs by positive sorting using anti-CD8 magnetic beads (Dynal AS, Oslo, Norway). More than 90% of the resulting cells were positive for CD8. For preparation of merozoites, *P falciparum* culture was treated with 10 μM E64 (Sigma Aldrich, St Louis, MO) to enrich schizont-stage parasites using the procedure described by Salmon et al.[22] After washing free of E64, the parasites were incubated for 3 to 6 hours in the presence of 50 μg/mL 5.2-OKT3 biscFv. $CD8^+$ T cells were added to the malaria culture when release of merozoites was detected. After incubation for one hour, cells were fixed with cold acetone/ethanol (6:4) on slide glass. After blocking with phosphate-buffered saline (PBS) containing 5% normal goat serum, the slides were incubated with fluorescein isothiocyanate (FITC)–conjugated anti-FLAG M2 mAb for one hour. The cells were washed 3 times with PBS, mounted with Vectashield (Vector Laboratories, Burlingame, CA) containing 4′6′-diamidino-2-phenylindole (DAPI), and observed by fluorescence microscopy.

Cytokine assay

The $PfMSP\text{-}1_{19}$ gene was cloned from *P falciparum* MAD20 strain, and inserted into the pBACgus-1 vector (Novagen) to construct a baculovirus transfer vector. Recombinant baculovirus expressing the $PfMSP\text{-}1_{19}$ gene, designated recBac-$PfMSP\text{-}1_{19}$, was constructed as routine procedure. Because recombinant $PfMSP\text{-}1_{19}$ protein ($rPfMSP\text{-}1_{19}$) was found to express a conformation-dependent epitope that is recognized by 5.2 mAb, the protein was purified by affinity chromatography on a 5.2 mAb column. Microtiter plates coated with 1 μg/mL purified $rPfMSP\text{-}1_{19}$ were incubated with scFv samples (50 μg/mL) for one hour at room temperature. Plates were washed with PBS, and a total of 2×10^5 PBMCs in 200 μL RPMI 1640 medium containing 10% fetal calf serum was added and incubated at 37°C under 5% CO_2. After 24 hours, supernatants of cultures were collected, and the levels of interleukin-2 (IL-2), interferon γ (IFN-γ), and tumor necrosis factor α (TNF-α) were measured by an enzyme-linked immunosorbent assay (ELISA; Genzyme, Boston, MA).

Inhibition and phagocytosis assay

ScFv samples were dialyzed against RPMI 1640 and then adjusted to a concentration of 200 μg/mL. Synchronization of blood-stage parasites was achieved after repeated sorbitol treatment.[23] Schizont-stage parasites were diluted with unparasitized red blood cells (RBCs) to give a final parasitemia of 1.0% at a 4% hematocrit. Then 50 μL scFv, 2×10^5 freshly prepared PBMCs in 50 μL complete media, and 100 μL parasitized RBCs were added in triplicate to wells of a 96-well plate. Each well contained 10 μg scFv, 2×10^5 PBMCs and a 2% hematocrit of RBCs at a 0.5% parasitemia. The experiment comprised the following: (1) parasite culture alone; (2) parasite culture with PBMCs; (3) parasite culture with scFv sample; and (4) parasite culture with scFv sample and PBMCs. All cultures were incubated at 37°C in 5% CO_2 for 24 or 72 hours. Supernatants from the cultures were collected and the levels of IL-2, IFN-γ, and TNF-α were measured by an ELISA (Genzyme). Growth inhibition was assessed after 72 hours of culture by microscopic examination of Giemsa-stained thin smears from each well, and percentage of parasitized RBCs was estimated by the counting of at least 1000 RBCs per smear. The mean ± SD parasitemia for each treatment was calculated from 3 replicate blood smears in each experiment. The specific growth inhibition (SGI) index was calculated according to Bouharoun-Tayoun et al[24] by the following equation: SGI of scFv alone = $100 \times [1-(\{\%\ \text{parasitemia with scFv sample}\}/\{\%\ \text{parasitemia with medium alone}\})]$; SGI of PBMCs = $100 \times [1-(\{\%\ \text{parasitemia with PBMCs}\}/\{\%\ \text{parasitemia with medium alone}\})]$; SGI of scFv plus PBMCs = $100 \times [1-(\{\%\ \text{parasitemia with PBMCs and scFv sample}\}/\{\%\ \text{parasitemia with scFv sample}\})/(\{\%\ \text{parasitemia with PBMCs}\}/\{\%\ \text{parasitemia with medium alone}\})]$.

Phagocytosis by monocytes was studied morphologically. After 24 or 72 hours of incubation of parasites, PBMCs, and scFv samples, the cells were resuspended with a pipettor to dislodge them from each well and cytocentrifuged thin smears were prepared using the total pellet, stained with Giemsa, and examined microscopically (× 1000). The phagocytosis rate was evaluated by counting the number of monocytes that had ingested one or more *P falciparum* merozoites per 500 monocytes in 2 separate experiments in triplicate. Differences between cultures were assessed by Student *t* test.

Results

Construction and expression of scFvs

We selected the anti–$PfMSP\text{-}1_{19}$ 5.2 mAb, derived originally against the Uganda Palo Alto strain of *P falciparum* (FUP), as a means of targeting blood-stage parasites because it recognizes an epitope within the 19-kDa C-terminal region of MSP-1 that is highly conserved in different *P falciparum* field isolates.[20,25] The 5.2 mAb itself has no direct effect on merozoite invasion or intraerythrocytic growth of the parasite in vitro. The V_H and V_L genes encoding 5.2 mAb were cloned from 5.2 hybridoma cells by RT-PCR. The *5.2 scFv* construct was inserted into the phage display vector pCANTAB5E. After transformation of *Escherichia coli* HB2151, the resultant phage pool (4×10^4) was screened with $rPfMSP\text{-}1_{19}$. Finally, we obtained one phage which had the capability of binding to $rPfMSP\text{-}1_{19}$ (data not shown). The V_H and V_L genes encoding 5.2 mAb were sequenced from this positive phage. The V_H and V_L genes encoding OKT3 mAb were cloned from OKT3 hybridoma cells by RT-PCR with primers designed from previous publication by Arakawa et al[19] The *5.2 scFv* and *OKT3 scFv* genes were connected through a $(Gly_4Ser)_3$ linker to construct the *5.2-OKT3 biscFv* gene. Figure 1 shows the nucleotide sequence and the predicted amino acid sequence of the *5.2-OKT3 biscFv* gene.

To facilitate high-level expression, 3 recombinant baculoviruses were generated harboring the following genes: (1) *5.2 scFv*, specific for *P falciparum* MSP-1; (2) *OKT3 scFv*, specific for human CD3; and

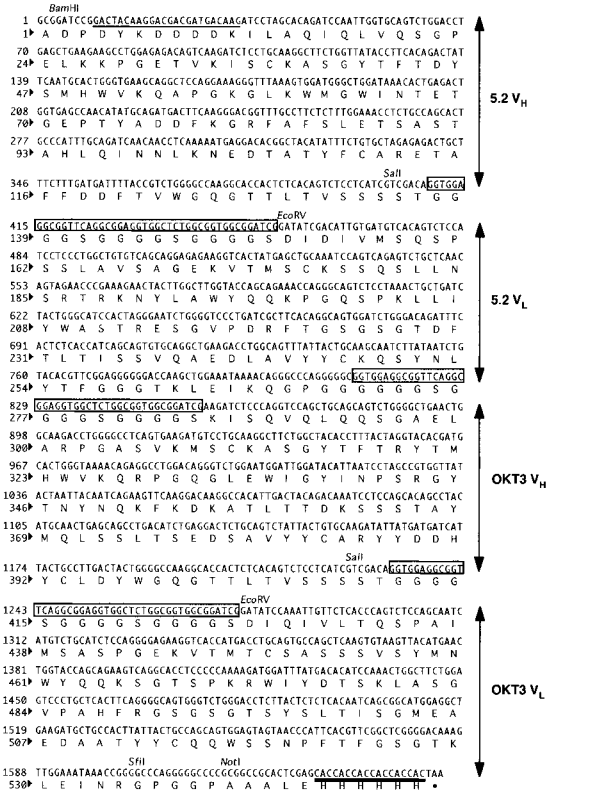

Figure 1. The nucleotide and deduced amino-acid sequences of 5.2-OKT3 biscFv. Sequences encoding $(Gly_4Ser)_3$ are boxed. The FLAG and His-tag sequences are underlined and bold underlined, respectively.

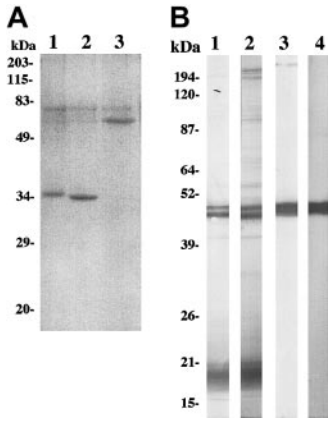

Figure 2. Expression and binding characteristics of 5.2-OKT3 biscFv. (A) Samples of scFv were purified from culture supernatants of recombinant baculovirus-infected cells by a Ni-NTA column as described in "Materials and methods." Purified 5.2 scFv (lane 1), OKT3 scFv (lane 2), and 5.2-OKT3 biscFv (lane 3) were separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (Nippon Bio-Rad Laboratories, Tokyo, Japan). (B) Native PfMSP-1 (lanes 1-2) and rPfMSP-1_{42} (lanes 3-4) were prepared for SDS-PAGE under nonreducing conditions and run on a 5% to 15% gradient gel. Proteins were transferred to Immobilon membranes (Millipore), probed with either 5.2 mAb followed by biotinylated goat anti–mouse IgG antibody (lanes 1,3) or 5.2-OKT3 biscFv followed by biotinylated mouse anti-FLAG M2 mAb (lanes 2,4).

(3) *5.2-OKT3 biscFv*, encoding a bispecific molecule consisting of 5.2 scFv fused to OKT3 scFv (Figure 2). The *5.2* and *OKT3 scFv* constructs each contain an intra-scFv $(Gly_4\text{-}Ser)_3$ linker connecting the C-terminal V_H segment with the N-terminal V_L segment. The *5.2-OKT3 biscFv* construct contains an additional intra-scFv $(Gly_4\text{-}Ser)_3$ linker connecting the C-terminal 5.2 V_L segment with the N-terminal OKT3 V_H segment. All 3 constructs contain the melittin signal leader peptide followed by the FLAG epitope tag at the N terminus and a histidine tag at the C terminus.

All constructs were expressed as secreted soluble proteins and purified by Ni-NTA chromatography from culture supernatants of High Five cells infected with recombinant baculoviruses. Each purified preparation appeared as a single band of the appropriate molecular size by SDS-PAGE (Figure 2A). As expected from the predicted amino acid sequences of scFvs, the apparent molecular weight of 5.2-OKT3 biscFv (77 kDa) was double that of 5.2 scFv (34 kDa) or OKT3 scFv (33 kDa).

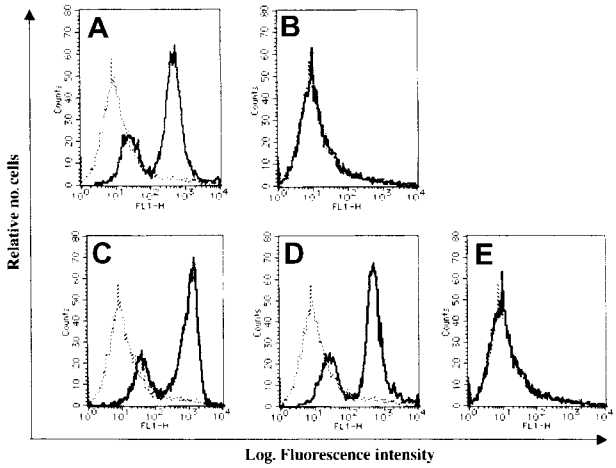

Figure 3. Flow cytometric analysis of the binding of human T cells by scFvs. Human T cells (2×10^6) were incubated with OKT3 mAb (A), 5.2 mAb (B), OKT3 scFv (C), 5.2-OKT3 biscFv (D), or 5.2 scFv (E), followed by anti-FLAG M2 secondary antibody. Cells were stained with FITC-conjugated goat anti–mouse IgG and analyzed by flow cytometry (solid line). Dotted line represents control cells incubated with the second and third step reagents alone. Data were obtained from 1 of 3 similar experiments.

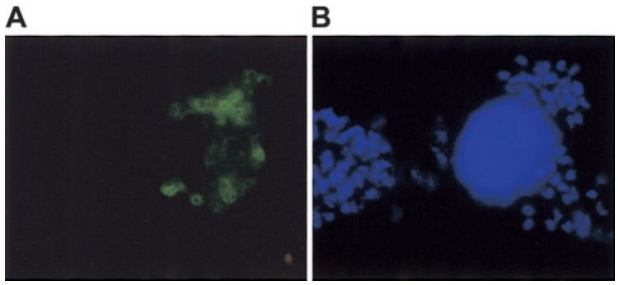

Figure 4. Bridge formation between $CD8^+$ T cells and merozoites by 5.2-OKT3 biscFv. Schizont-stage parasites were enriched by treatment with E64 as described in "Materials and methods." To release merozoites from its parasitophorous vacuole, E64 was removed completely from culture. The parasites were incubated for 3 to 5 hours in the presence of 50 μg/mL 5.2-OKT3 biscFv. When merozoites were detected in the culture, $CD8^+$ T cells were added and incubated for one hour. (A) Bridge formation between $CD8^+$ T cells and merozoites was detected by indirect immunofluorescence staining with FITC-conjugated anti-FLAG M2 mAb. (B) The same image is shown with DAPI staining and photographed through a fluorescence microscope. The nuclei of $CD8^+$ T cells and merozoites were stained in blue. Original magnification, × 1000.

Binding properties of 5.2-OKT3

The binding properties of 5.2-OKT3 biscFv to PfMSP-1 and CD3 were shown by immunoblot and fluorescent-activated cell sorting (FACS) analysis, respectively. As shown in Figure 2B, 5.2-OKT3 biscFv specifically reacted with 19-kDa and 42-kDa purified, parasite PfMSP-1 (lane 2) of the *P falciparum* FUP strain as well as 42-kDa rPfMSP-1_{42} (lane 4). The specificity of 5.2-OKT3 biscFv was indistinguishable from that of the parent 5.2 mAb (lanes 1,3). Figure 3 shows that OKT3 scFv (panel C) and 5.2-OKT3 biscFv

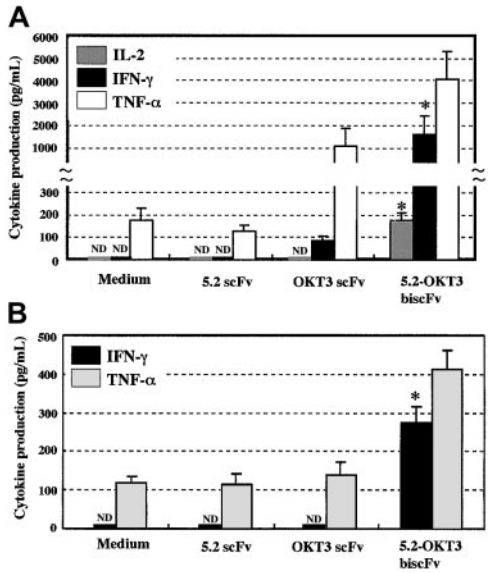

Figure 5. Production of IL-2, TNF-α, and IFN-γ from mononuclear cells stimulated with scFvs. (A) ScFvs (100 μg/mL) were preincubated with rPfMSP-1_{19}–coated 96-well plates. After washing, PBMCs (2×10^5/well) were added and cultured for 24 hours. Culture supernatants were harvested and assayed for production of IL-2, TNF-α, and IFN-γ by a sandwich ELISA. (B) Highly synchronous blood-stage cultures of *P falciparum* at an initial parasitemia of 1% were cocultured with PBMCs (2×10^5/well) and scFvs (50 μg/mL) for 24 hours in a 96-well plate. Culture supernatants were assayed as described in panel A. Results are presented as the means ± SDs from 3 separate experiments. *$P < .01$ compared with 5.2 scFv and OKT3 scFv. ND indicates not detectable.

Table 2. SGI assay with scFv samples plus PBMCs

	Medium		5.2 scFv		OKT3 scFv		5.2-OKT3 biscFv	
PBMCs*	−	+	−	+	−	+	−	+
Parasitemia, %	2.63 ± 0.54	2.32 ± 0.19	2.32 ± 0.19	2.41 ± 0.59	2.39 ± 0.41	2.16 ± 0.60	2.79 ± 0.63	1.56 ± 0.29
SGI, %	0	10.3 ± 6.3†	16.1 ± 6.6‡	−24.8 ± 15.0§	8.6 ± 2.9‡	−1.7 ± 13.9§	−6.0 ± 2.0‡	36.8 ± 3.6§

For SGI assay, the results are expressed as the final parasitemia obtained after 72 hours of culture and as the SGI, calculated as indicated in "Materials and methods." All values represent the mean ± SD of 3 separate experiments. Percentages of SGI with negative values represent enhancement of parasite growth.
*+ and − indicate scFv samples with or without PBMCs, respectively.
†SGI of PBMCs.
‡SGI of scFv.
§SGI of scFv plus PBMCs.

(panel D) were comparable to OKT3 mAb (panel A) in binding to human T cells, as shown by their increased fluorescence intensity compared with 5.2 mAb (panel B) or 5.2 scFv (panel E). These results clearly demonstrate that 5.2-OKT3 biscFv retains the binding properties of both parental antibodies.

We examined whether 5.2-OKT3 biscFv can form a bridge between merozoites and T cells. To facilitate the observation of bridge formation, $CD8^+$ T cells were isolated from PBMCs as effector cells capable of binding to OKT3. At the moment that schizont-stage parasites rapidly rupture to release merozoites from their parasitophorous vacuole following removal of E64, human $CD8^+$ T cells were added to the culture in the presence of 5.2-OKT3. DAPI staining showed merozoites were agglutinated on the surface of $CD8^+$ T cells. The binding sites exhibited intense fluorescence, whereas no positive signal was detected in schizont-stage parasites. This result indicates that $CD8^+$ T cells can bind to free merozoites through 5.2-OKT3 biscFv.

Immunologic characteristics of 5.2-OKT3 scFv

To investigate whether the bridge formation shown in Figure 4 could activate T cells, we examined cytokine production following bridge formation between rPbMSP-1_{19} and PBMCs through 5.2-OKT3 biscFv. rPbMSP-1_{19} was immobilized to concentrate the binding complex of rPbMSP-1_{19} and 5.2-OKT3 biscFv in a well. Unbound scFvs were washed away to confirm the specific binding of 5.2-OKT3 biscFv and rPbMSP-1_{19}. PBMCs were added and cultured in the plate for 24 hours. The production of IL-2, IFN-γ, and TNF-α in culture supernatants was measured by an ELISA assay. Figure 5A shows that production of these cytokines was specifically induced by 5.2-OKT3 biscFv. Regardless of the absence or presence of soluble rPfMSP-1_{19}, both OKT3 scFv and 5.2-OKT3 biscFv could activate PBMCs to induce approximately 10% to 30% of cytokine (TNF-α but not IFN-γ) produced by bridge formation through 5.2-OKT3 biscFv (data not shown). Presumably, this low level of activation is due to direct binding of 5.2-OKT3 biscFv or OKT3 scFv to T cells. This result is consistent with previous reports that cross-linking of OKT3 is more effective to activate T cells compared with the soluble form.[26,27]

Functional activity of 5.2-OKT3 biscFv

To assess the potential therapeutic value of 5.2-OKT3 biscFv against *P falciparum* blood-stage parasites, we measured cytokine production, *P falciparum* growth inhibition and parasite/merozoite phagocytosis in cocultures of PBMCs, and *P falciparum* blood stages in the presence of 5.2-OKT3 biscFv. Similar to the result in Figure 5A, significant levels of IFN-γ and TNF-α production were induced in the cocultures when 5.2-OKT3 biscFv was present (Figure 5B). In contrast, high levels of TNF-α production were induced by PBMCs even in the absence of any biscFv, consistent with previous work in which high levels of TNF-α activity were detected when monocytes were cocultured with *P falciparum* schizont-stage parasitized erythrocytes that subsequently ruptured.[28] No IL-2 production was detected in this experiment (data not shown).

As shown in Table 2, 5.2-OKT3 biscFv in cocultures of *P falciparum* and PBMCs caused inhibition of parasite growth at an SGI of 36.8%, whereas 5.2 scFv and OKT3 scFv cocultured with PBMCs enhanced parasite growth as shown by negative SGIs of −24.8% and −1.7%, respectively. PBMCs alone and 5.2 scFv alone showed very slight inhibition (SGI = 10.3% and 16.1%, respectively). In addition to SGI, we observed many monocytes actively phagocytosing the parasites (Figure 6Bi) and the presence of many agglutinated clusters of merozoite phagocytosis (Figure 6Bii) under microscopic examination of Giemsa-stained smears of monocyte–*P falciparum* cocultures containing 5.2-OKT3 biscFv. Some fields contained parasites with abnormal morphology resembling dying intracellular parasites or "crisis forms" (Figure 6Biii). These abnormal parasites may have been produced as a result of the release of soluble mediators by activated monocytes. Figure 6 shows the rate of phagocytosis after 24 or 72 hours of culture. The presence of 5.2-OKT3 biscFv in cocultures of *P falciparum* and PBMCs promoted a significant increase in merozoite phagocytosis

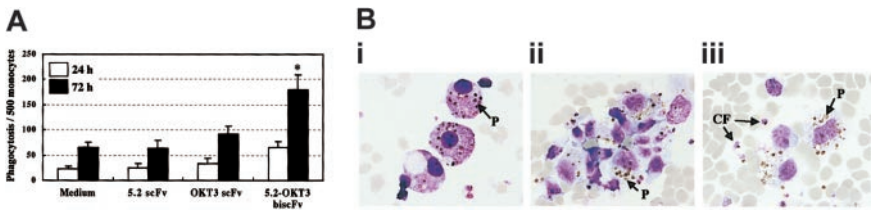

Figure 6. Phagocytosis rate in the presence of scFvs. (A) Highly synchronous blood-stage cultures of *P falciparum* were cocultured with PBMCs (2×10^5/well) in the presence or absence of scFvs (50 μg/mL) for 24 or 72 hours in a 96-well plate. The rate of phagocytosis was evaluated by counting the number of cells ingesting one or more *P falciparum* merozoites per 500 monocytes. Results are presented as the means ± SDs of 3 separate experiments performed in triplicate. *$P < .05$ compared with 5.2 scFv and OKT3 scFv. (B) Also shown are examples of the phagocytosis. Photomicrographs of Giemsa-stained smears of in vitro *P falciparum* cocultured with PBMCs in the presence of 5.2-OKT3 biscFv. Many clusters of parasites phagocytosed by monocytes were observed when *P falciparum* was cultured with 5.2-OKT3 biscFv and PBMCs. (i) Monocytes actively phagocytosing parasites. (ii) Agglutinated clusters of merozoite phagocytosis. (iii) Monocytes phagocytosing parasites and crisis forms of parasites. CF indicates crisis form; P, malaria pigment. Original magnification, × 1000.

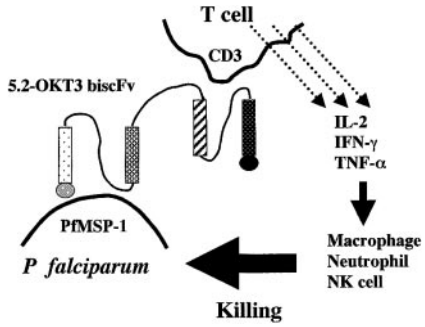

Figure 7. Schematic representation of proposed mechanism of 5.2-OKT3 biscFv-mediated cellular inhibition of parasite growth.

after 72 hours of culture ($P < .05$), compared with PBMCs in the presence of 5.2 scFv or OKT3 scFv.

Discussion

In the present study, we constructed 5.2-OKT3 biscFv to design a potentially immunotherapeutic ligand for blood-stage malaria parasites. We found that 5.2-OKT3 biscFv possessed specific binding activities for PfMSP-1_{19} on the surface of merozoites and for CD3 on human T cells. Binding of 5.2-OKT3 biscFv to human T cells induced an increase in production of IFN-γ and TNF-α in vitro. Additionally, 5.2-OKT3 biscFv promoted merozoite phagocytosis and the induction of "crisis forms" in cocultures containing PBMCs. Approximately 35% of monocytes contained malaria pigments and other parasite debris in the presence of 5.2-OKT3 biscFv. Previous in vivo and in vitro studies have suggested that IFN-γ and TNF-α play important roles in protecting against blood-stage *P falciparum* in humans.[29] In mice, IFN-γ regulates the production of the opsonizing or cytophilic and complement-fixing immunoglobulin G2a (IgG2a) and IgG3 involved in the phagocytosis of particulate micro-organisms and antibody-dependent cellular cytotoxicity. Macrophages, neutrophils, and natural killer (NK) cells, the main cell effectors of nonspecific, innate immunity, may be modulated by IFN-γ and TNF-α to induce degenerating parasites, referred to as "crisis forms," within the host erythrocytes.[30,31] Therefore, it is possible that T-cell activation and cytokine production induced by 5.2-OKT3 biscFv, which are brought into close contact with merozoites via bridge formation, result in merozoite phagocytosis by monocytes.

Based on our data and on mechanisms proposed by others for biscFv biologic activity, we propose that bridge formation between parasites and T cells through 5.2-OKT3 biscFv provides the initial trigger for the parasite clearance (Figure 7). T cells are stimulated with binding of 5.2-OKT3 biscFv to CD3 and release soluble mediators like IL-2, IFN-γ, and TNF-α able to act on nearby cells. These soluble mediators may activate effector cells such as macrophages, neutrophils, and NK cells to kill the blood-stage parasites by phagocytosis and intracellular digestion or through the release of toxic substances such as nitrous oxide.

Druilhe and coworkers[24,32] found that human IgG of protected hyperimmune subjects inhibits parasite growth (SGIs = 60, approximately 90%) in the presence of monocytes (2×10^5/well) via antibody-dependent cellular inhibition (ADCI), whereas antibodies alone either had no effect or enhanced parasite growth.[24,32] Parasite growth inhibition (SGI = 36.8%) observed in the presence of 5.2-OKT3 biscFv and PBMCs may or may not occur by similar mechanisms as ADCI. Because the main purpose of the present study was to address the potential usefulness of biscFvs in malaria patients, we used freshly prepared PBMCs (2×10^5/well) rather than purified T cells and monocytes to simulate naive culture conditions without prestimulation. Consequently, the number of monocytes responsible for phagocytosis might be insufficient to completely inhibit parasite growth inhibition. It is also possible that T cells and/or monocytes used in the present studies may have not been fully activated under the current culture conditions. Additional signals may be required to achieve efficient killing of ingested parasites. Studies using an antitumor scFv have identified a requirement for a B7-mediated costimulatory signal along with TCR-mediated binding for optimal T-cell activation.[33] More extensive studies are required to define the requirements for optimal interactions between T cells, mononuclear effector cells, and 5.2-OKT3 biscFv.

BiscFv-mediated selective immunotherapy has been used to retarget T-cell cytotoxicity against tumor cells, and some of these molecules are now being evaluated in clinical trials.[7-9] An application of this mechanism for the control of infectious diseases is the use of a biscFv composed of OKT3 scFv and CD4 to target and kill HIV-infected cells.[10,11] These immune-activating properties of biscFv may provide a means for enhancing biscFv therapy via several distinct mechanisms. In addition to the current example of 5.2-OKT3 biscFv as an immunotherapeutic drug, we have recently constructed an immunotoxin composed of 5.2 scFv and an antimalaria toxin. The potential parasitocidal effect of this immunotoxin on parasite growth inhibition is now under investigation.

The present study demonstrates that 5.2-OKT3 biscFv offers distinct advantages as an antimalarial therapeutic: (1) the PfMSP-1_{19} epitope recognized by 5.2-OKT3 biscFv is highly conserved and thus is suitable as a target molecule; (2) 5.2-OKT3 biscFv cross-linking to T cells and malaria parasites results in the production of IFN-γ and TNF-α in a manner that is unrestricted by major histocompatibility complex (MHC) haplotype; and (3) monocytes and macrophages activated by IFN-γ and TNF-α may be capable of the elimination of erythrocytic forms of malaria parasites from the circulation. In summary, 5.2-OKT3 biscFv appears to be a good candidate for immunotherapeutic elimination of *P falciparum* unrestricted by malaria antigen diversity and MHC-controlled responsiveness in a genetically diverse human population.

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