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Epitope-Specific Regulation of Immunoglobulin Class Switching in Mice Immunized with Malarial Merozoite Surface Proteins

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Antibodies that bind to Fc receptors and activate complement are implicated in the efficient control of pathogens, but the processes that regulate their induction are still not well understood. To investigate antigen-dependent factors that regulate class switching, we have developed an in vivo model of class switching to immunoglobulin G2b (IgG2b) using the malaria antigen *Plasmodium falciparum* merozoite surface protein 2 (MSP2). C57BL/6 mice were immunized with recombinant proteins representing discrete domains of MSP2, and a T-cell epitope (C8) was identified within the conserved C terminus of the protein that preferentially induces IgG2b antibodies. The ability of C8 to induce IgG2b is ablated in both homozygous gamma interferon-negative and interleukin 10-negative mice. The IgG2b-inducing properties of C8 override the IgG1-inducing properties of both the fusion protein partner, glutathione *S*-transferase, and the adjuvant. Furthermore, when attached to other proteins that normally induce IgG1 responses, C8 induces a switch to IgG2b secretion. This is the first description of a defined T-cell epitope that drives specific IgG2b subclass switching, and our data offer proof of the concept that chimeric vaccines incorporating specific T-cell "switch epitopes" might be used to enhance qualitative aspects of the antibody response.

The ability of antibodies to control or clear pathogens depends on their specificity, their avidity for antigen, and their isotype or subclass. Antibodies which bind Fc receptors and mediate antibody-dependent cytotoxicity, growth inhibition, or phagocytosis (so-called "cytophilic" antibodies) have repeatedly been shown to be highly effective at clearing blood stage malaria parasites, both in humans (14, 43) and in mice (36, 38, 55). In humans, immunoglobulin G3 (IgG3) is especially valuable in this regard (4, 19), and the presence of IgG3 to various merozoite-associated antigens has been linked to protective immunity in seroepidemiological studies (10, 15, 35, 51, 56).

A number of *Plasmodium falciparum* antigens (5, 8, 9, 56) have been shown to preferentially induce IgG3 in humans; the first of these antigens to be characterized was merozoite surface protein 2 (MSP2) (42, 52), but similar observations have now been made for a polymorphic N-terminal region (block 2) of MSP1 (9) and for MSP3 (9, 37), MSP4 (57), and MSP7 (56). This bias towards IgG3 production to protein antigens is highly unusual (23) and suggests that something in the interaction of these proteins with the human immune system very efficiently triggers IgG3 class switching. Identifying antigen-specific elements that regulate immunoglobulin class switching may allow such elements to be incorporated into synthetic, subunit vaccines in order to induce optimal IgG subclasses and highly efficient effector mechanisms.

Subclass switching, in which variable heavy-chain (V_H) genes combine with different constant heavy-chain (C_H) genes to produce antibodies of a single antigen specificity but with differing Fc regions and thus differing functions, is an integral part of B-cell maturation, and a key step in this process is transcription through specific C_H gene switch regions and excision of C_H genes upstream of the C_H gene to be expressed (11, 47). A variety of stimuli, including lipopolysaccharide (LPS) and signaling via CD40-CD154 and various cytokines, have been shown to induce various patterns of class switching in B cells in model systems, but much less is known about the regulation of class switching in vivo in response to specific antigens. In particular, the reasons why some antigens preferentially induce antibodies of certain isotypes or subclasses are poorly understood.

We have used *P. falciparum* MSP2 as a model antigen to explore antigen-specific class switching in vivo. MSP2 is a highly polymorphic, glycosylphospatidylinositol-anchored protein expressed on trophozoites, schizonts, and merozoites (12, 21, 46). The amino (23-amino-acid) and carboxyl (56-amino-acid) termini of MSP2 are highly conserved; internal to these conserved regions, serogroup-specific sequences flank highly polymorphic central sequences which contain repeated amino acid motifs (Fig. 1). MSP2 variants can be grouped into two major serogroups, type A (typified by cloned isolate 3D7) and type B (e.g., isolates FCR3 and HB3) (21, 45); certain B-cell epitopes appear to be conserved, giving rise to antigenic cross-reactivity within each family (20, 24). Thus, cross-reactive epitopes within dimorphic or polymorphic sequences, or conserved sequences within the N and C termini of the protein (Con-N and Con-C, respectively), may explain the apparent ability of all MSP2 serotypes to drive IgG3 class switching. The polymorphic and dimorphic regions of the molecule are im-

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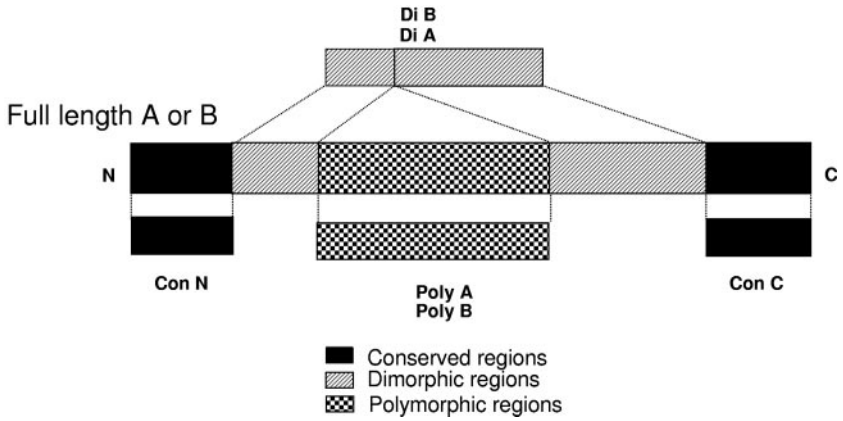

FIG. 1. Schematic showing the predicted protein structure of MSP2 and the derivation of the recombinant proteins. Filled blocks indicate sequences that are conserved among all *P. falciparum* isolates. Hatched blocks indicate dimorphic sequences which differ between A family and B family proteins but which are conserved within families. Checkered blocks indicate the highly polymorphic central region of the molecule, which contains tandemly repeated amino acid sequences. The recombinant proteins representing the dimorphic sequences (Di-A and Di-B) comprise the N-terminal dimorphic sequence fused to the C-terminal dimorphic sequence with exclusion of the intervening polymorphic sequence.

munodominant for B cells, whereas the invariant N and C termini induce very poor antibody responses in immunized mice (30) or in humans under conditions of natural exposure to infection (20, 52, 53a, 54). By contrast, human and murine T cells respond to epitopes within both conserved and variable sequences of the molecule (40, 41, 53).

In order to explore the antigen-specific effects that lead to highly directed class switching to cytophilic IgG antibodies, we have immunized C57BL/6 mice with recombinant proteins representing full-length, polymorphic, dimorphic, and conserved sequences of MSP2 attached to a conserved fusion protein partner, *Schistosoma japonicum* glutathione *S*-transferase (GST). We find that mice, like humans, develop skewed IgG antibodies to MSP2 with the highly cytophilic IgG2b subclass (which is typically a minor component of the murine antibody response to protein antigens and which shares many features with human IgG3), dominating the response. Thus, although human and mouse gamma globulin genes are not true orthologues, it seems likely that control of the class-switching machinery may be evolutionarily conserved and that this mouse model might provide insight both into the mechanisms underlying the ability of MSP2 to promote particular patterns of IgG subclass switching and into the requirements for antigen-specific class switching in general.

MATERIALS AND METHODS

Animals and immunizations. Female C57BL/6 mice (Harlan, Oxon, United Kingdom), homozygous gamma interferon-negative (IFN-$\gamma^{-/-}$) mice on a C57BL/6 background (originally from Jackson Laboratories, Maine), and homozygous interleukin 10-negative (IL-$10^{-/-}$) mice on a C57BL/6 background (originally from the Institut für Genetik, Köln, Germany) were maintained in positive-pressure isolators, as described previously (32). Mice (8 to 10 weeks of age; six per group) were immunized intraperitoneally with 10 μg of recombinant protein emulsified 1:1 in RIBI adjuvant (monophosphoryl lipid A plus synthetic trehalose dicorynomycolate; Sigma, Poole, Dorset, United Kingdom); three immunizations were given at 2-week intervals. Blood was collected prior to each immunization, and serum was stored for antibody analysis. Two or four weeks after the final immunization; serum was collected for serology and spleen cells were harvested.

Expression and purification of recombinant proteins. Full-length MSP2 proteins representing serogroup A (MSP2A proteins) and serogroup B (MSP2B proteins), expressed as hexa-His fusion proteins in *Escherichia coli*, were kindly provided by Robin Anders, La Trobe University, Australia (23a). Discrete domains of MSP2 were produced in *E. coli* as fusion proteins with the C-terminal region of GST (44) using the pGEX expression system (Amersham Pharmacia Bioscience, Little Chalfont, United Kingdom). The production and validation of proteins representing the dimorphic sequences of serogroup A (Di-A) and serogroup B (Di-B) and polymorphic sequences from each serogroup (Poly-A and Poly-B) have been described previously (24). Conserved 5′ and 3′ sequences of the MSP2 gene (Con-N], ~320 bp, and Con-C, ~325 bp) were amplified using specific primers (CN5′ [CCAGTACCAGTAGGAGGC] and CN3′ [GAAGAG AATTATATGAATATGGC]), ligated into pGEX-3, validated by DNA sequencing (44), and expressed in *E. coli*. The molecular mass (35 kDa) of the expressed proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and their reactivity with MSP2-specific antibodies was confirmed by immunoblotting (data not shown). Finally, sera from mice immunized with Con-N and Con-C were shown by immunoblotting to recognize a band of approximately 46 kDa, representing the full-length A protein (data not shown).

GST-$MSP1_{19}$ ($MSP1_{19}$) and GST control antigens were purified from *E. coli* clones provided by A. Holder (NIMR, United Kingdom) (7).

A schematic of the MSP2 proteins is shown in Fig. 1.

Peptides and peptide conjugation. Eight overlapping 20-mer peptides, spanning the entire conserved C terminus of *P. falciparum* MSP2, were synthesized by conventional solid-phase techniques on a Pioneer peptide synthesis system (PerSeptive Biosystems, Framingham, MA) (Table 1) and purified by reverse-phase high-performance liquid chromatography. Peptides (C8 and L) were conjugated to bovine serum albumin (BSA; Pierce Biotechnology, Inc., Tattenhall, United Kingdom) and to GST-$MSP1_{19}$ activated with sulfo-MBS (*m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester) (Pierce Biotechnology, Inc.) according to the manufacturer's protocol.

Antibody detection by ELISA. IgG and IgG subclass antibodies were measured by direct enzyme-linked immunosorbent assay (ELISA) as described previously (51). Microtiter plates were coated with antigen (1 μg/ml), washed, and blocked, and serum (serially diluted in phosphate-buffered saline–0.05% Tween 20 with 3% nonfat milk) was added to duplicate wells. After incubation overnight at 4°C, the plates were washed, incubated with horseradish peroxidase-conjugated antibodies to the murine IgG or IgG subclass (Southern Biotechnology), and developed with *o*-phenylenediamine (Sigma, Poole, Dorset, United Kingdom) and hydrogen peroxide. The reaction was stopped after 10 min with 50 μl/well 2 M H_2SO_4, and the optical density (OD) was measured at 492 nm.

As titers of antibodies of different subclasses are difficult to compare directly (as the secondary reagents used for their detection may differ in avidity), we have used a subclass index as a semiquantitative measure of the relative concentrations of the different subclasses. The subclass index is the reciprocal of the midpoint titer for IgG1 antibodies divided by the reciprocal of the midpoint titer for IgG2b antibodies. Midpoint titers were defined as the midpoint of the fitted sigmoid obtained from individual mouse serum titrations.

In vitro spleen cell stimulation. Single-cell suspensions of mouse splenocytes were suspended in RPMI 1640 with 5% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (all from Invitrogen, Paisley, United Kingdom) and incubated (10^6 cells/well) at 37°C in 5% carbon dioxide. Recombinant proteins were added to triplicate wells at a final concentration of 1 μg/ml, synthetic peptides at 10 μg/ml, LPS at 5 μg/ml, and concanavalin A

TABLE 1. Median antibody titers after three immunizations of C57BL/6 mice with recombinant MSP2 and $MSP1_{19}$ proteins

Immunizing antigen	Median midpoint titer (10^5) (95% CI)[a]					Mean IgG1/IgG2b ratio[b]	*P* (MSP2 vs $MSP1_{19}$)
	Total IgG	IgG1	IgG2a	IgG2b	IgG3		
$MSP1_{19}$	8.34 (2.19)	5.02 (1.97)	0.08 (0.41)	3.89 (0.52)	0.05 (0.13)	2.89	
Serogroup A							
Full length (MSP2A)	9.60 (1.79)	6.34 (1.66)	0.83 (0.44)	2.95 (0.37)	0.43 (0.18)	4.69	0.008
Di-A	17.8 (3.57)	2.55 (1.43)	0.18 (0.09)	12.0 (3.09)	0.11 (0.08)	0.32	0.007
Poly-A	0.64 (0.30)	0.19 (0.12)	0.04 (0.02)	0.47 (0.12)	0.03 (0.02)	0.51	0.011
Serogroup B							
Full length (MSP2B)	19.3 (4.55)	11.8 (2.58)	0.78 (0.41)	3.91 (1.38)	0.51 (0.13)	5.92	0.014
Di-B	3.84 (1.89)	0.76 (0.47)	0.04 (0.01)	2.50 (1.25)	0.03 (0.03)	0.34	0.009
Poly-B	5.5 (0.63)	1.11 (0.88)	0.07 (0.05)	4.37 (1.25)	0.03 (0.01)	0.44	0.008
Con-C	2.61 (0.28)	0.18 (0.12)	0.08 (0.06)	2.22 (0.63)	0.06 (0.03)	0.18	0.006
Con-N	0.64 (0.11)	0.45 (0.08)	0.02 (0.01)	0.42 (0.10)	0.01 (0.01)	1.46	0.064

[a] There were six mice per group. 95% CI, 95% confidence interval.
[b] Calculated as the mean for each mouse. Shown are the mean ratios of IgG1 to IgG2b (the IgG1/IgG2b index) and comparisons between the IgG1/IgG2b index for MSP2 proteins and the index for $MSP1_{19}$ (Wilcoxon rank sum tests).

(Sigma, Poole, Dorset, United Kingdom) at 5 μg/ml. Supernatants were collected after 24 h and 3 and 5 days and stored at −70°C, and cell proliferation was determined by 18-h ^3H incorporation on day 6. The stimulation index was calculated as the geometric mean number of cpm of stimulated wells divided by the geometric mean number of cpm of the relevant control wells.

Cytokine analysis. Bead-based multiplex analysis (Bio-Rad, Herts, United Kingdom) was used to determine concentrations of tumor necrosis factor alpha, IFN-γ, IL-10, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, and granulocyte-macrophage colony-stimulating factor in spleen cell supernatants. Plates were analyzed by a Luminex 100 apparatus (Luminex Corp, Austin, TX), and mean fluorescence intensities were converted into cytokine concentrations using five-parameter fit standard curves.

Statistical analysis. Data were analyzed using STATA 8 (StataCorp, Austin, TX). Median and 95% confidence intervals were generated from midpoint titers, and statistical significance was determined using nonparametric (Wilcoxon rank sum) tests.

To compare the relative abundances of IgG1 and IgG2b among groups, subclass indices or ratios of the midpoint IgG1 titer to the midpoint IgG2b titer were calculated, and statistical significance was determined using nonparametric (Wilcoxon rank sum) tests.

RESULTS

The conserved C terminus of MSP2 induces IgG2b responses in C57BL/6 mice. Mice were immunized with recombinant $MSP1_{19}$, with MSP2 fusion proteins, and with GST alone. Serum was collected prior to each immunization and 2 weeks after the third immunization. Antibodies (total IgG and IgG subclasses) to the immunizing antigen and to the GST control protein were determined by midpoint titration. Data are shown (Fig. 2) for the conserved proteins and MSP2 serogroup A proteins; similar observations were made for equivalent serogroup B proteins (Table 1).

Total IgG titers increased steadily over the course of immunization for all groups of mice (Fig. 2a); all the proteins tested were highly immunogenic, inducing antibody titers of >1:50,000 after three immunizations. However, Di-A induced maximal IgG titers that were approximately twice those induced by $MSP1_{19}$ and MSP2A and 10- to 30-fold higher than those induced by Poly-A, Con-C, and Con-N. Mice immunized with GST plus adjuvant or adjuvant alone did not produce anti-MSP2 antibodies (data not shown) and made only low titers of pure IgG1 antibodies to GST itself (Fig. 2b).

Two weeks after the first immunization, antibodies to $MSP1_{19}$ and most of the MSP2 recombinant proteins were of mixed subclasses, with IgG1 and IgG2b tending to dominate over IgG2a and IgG3 (Fig. 2c to h). However, the MSP2 Con-C protein immediately generated a high-titer IgG2b response, with titers of IgG1 being similar to the background (Fig. 2g). After the second and third immunizations, IgG1 antibodies dominated the response to full-length MSP2 proteins, with IgG1 titers being fourfold higher than those of IgG2b (Table 1). Approximately equivalent titers of IgG1 and IgG2b were produced in response to Con-N and to $MSP1_{19}$. However, after three immunizations, IgG2b antibodies became dominant in response to dimorphic and polymorphic proteins; this is also evident from the IgG1 to IgG2b index (Table 1). In all cases, titers of IgG2a and IgG3 rose in parallel and were 10- to 100-fold lower than IgG1 and IgG2b titers. Importantly, the early IgG2b response to Con-C was maintained throughout the immunization period (Fig. 2g), and after the third immunization, IgG2b titers were 10-fold higher than IgG1 titers (subclass index, 0.18) (Table 1).

Thus, the conserved C terminus of MSP2 appears to specifically promote the induction of IgG2b antibodies, even in the presence of an adjuvant and a carrier protein that normally induce a very polarized IgG1 response.

T cells from immunized mice recognize an epitope in the conserved C terminus of MSP2 by lymphoproliferation and cytokine production. We considered that the most likely explanation for differential IgG induction by the various recombinant antigens was that the antigens differed in the nature of the T-cell help that they induced. In particular, we hypothesized that the Con-C protein included a T-cell epitope that was particularly effective at inducing class switching to IgG2b. Thus, we have analyzed lymphoproliferative and cytokine responses of spleen cells from immunized mice following restimulation in vitro with the immunizing antigen for periods of up to 6 days. In order to identify T-cell epitopes within the Con-C protein, spleen cells were also restimulated with overlapping 20-mer peptides representing the entire Con-C sequence (Table 2).

Lymphoproliferative responses. Spleen cells from mice immunized three times with full-length MSP2A made strong pro-

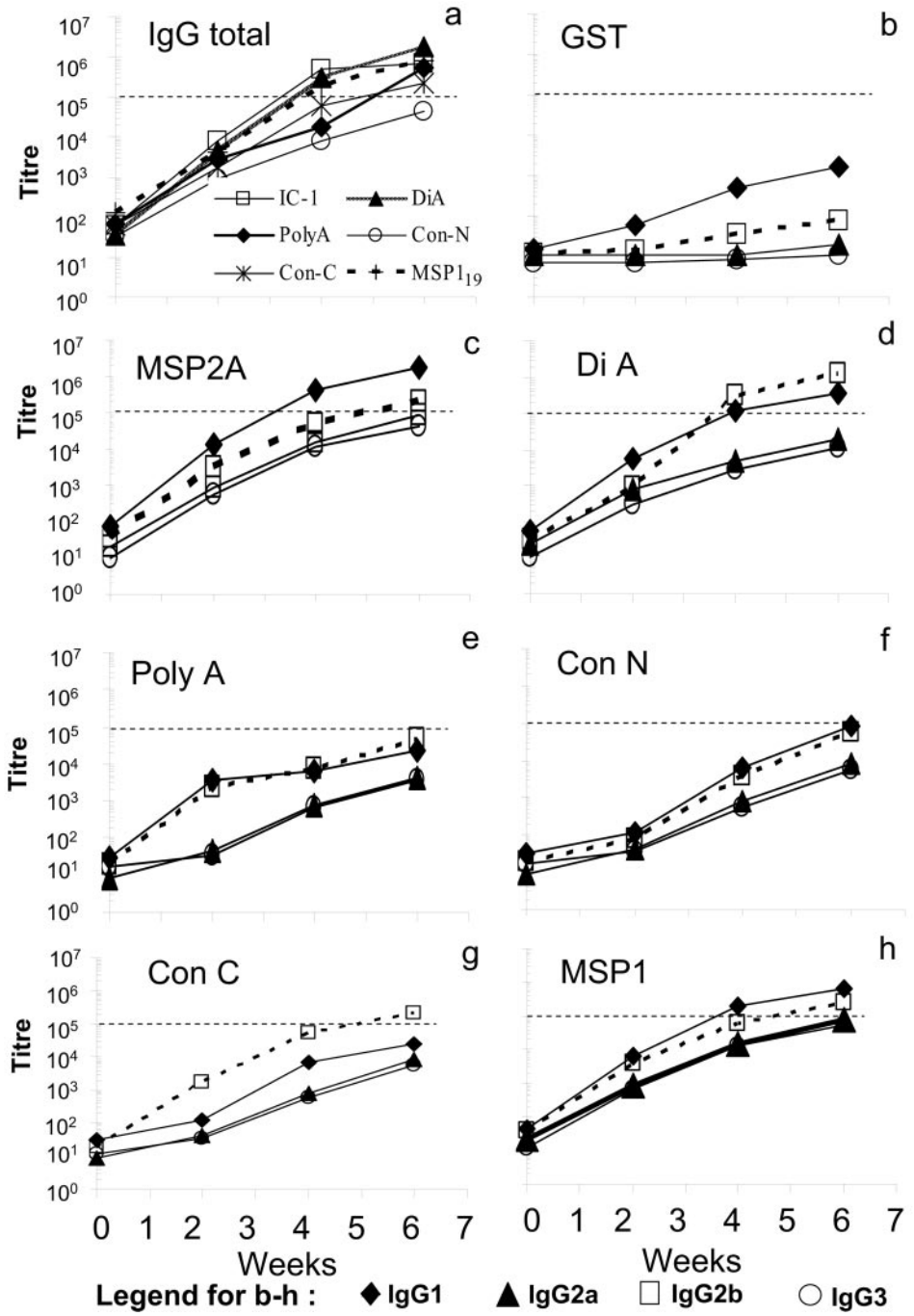

FIG. 2. Total IgG and IgG subclass antibody responses to MSP1$_{19}$ and MSP2 (serogroup A) proteins in C57BL/6 mice. Median midpoint titers of IgG and IgG subclasses (measured by ELISA) are shown for sera (six mice per group) collected prior to immunization and 2 weeks after each immunization with GST, MSP2, or MSP1$_{19}$ proteins. Horizontal dotted lines indicate a titer of 10^5. (a) Total IgG titers. Sera were tested against the same protein that was used for immunization. (b) IgG subclass titers to GST alone. Geometric mean titers are shown for all groups of mice combined (n = 36). (c to h) IgG subclass titers for mice immunized with different recombinant proteins. Sera were tested against the same protein that was used for immunization. (c) Full-length MSP2-His$_6$ (MSP2A); (d) Di-A–GST; (e) Poly-A–GST; (f) Con-N; (g) Con-C–GST; (h) unrelated antigen-GST (MSP1$_{19}$).

liferative responses to both MSP2A and the Con-C protein but made no responses to the unrelated MSP1$_{19}$ antigen (Table 2). Similarly, cells from mice immunized with the Con-C protein made proliferative responses to MSP2A and to Con-C but not to MSP1$_{19}$. Control mice, immunized with MSP1$_{19}$, made varied responses to MSP1$_{19}$ itself and occasional weak responses to MSP2 antigens. Interestingly, none of the mice made strong proliferative responses to the GST fusion protein, indicating that GST itself is not a potent inducer of T-cell help.

The only Con-C peptide that was reliably recognized by cells from MSP2-immunized mice was C8, representing the most carboxyl-terminal 20 amino acids of the MSP2 protein, al-

TABLE 2. Lymphoproliferative responses[a] of splenocytes from immunized C57BL/6 mice to recombinant protein and synthetic peptide antigens

Immunizing antigen	SI for indicated antigen for restimulation of spleen cells[b]												
	Poly-A	Con-C	$MSP1_{19}$	C1	C2	C3	C4	C5	C6	C7	C8	L	GST
MSP2A	**4.4**	**4.4**	1.3	0.5	0.9	0.8	1.1	0.5	1.8	1.1	**2.0**	1.1	1.1
	8.1	**5.9**	1.0	0.4	1.1	1.5	0.4	0.3	1.3	**4.5**	**7.4**	1.3	1.7
	5.1	**4.0**	1.2	0.7	1.0	1.3	1.1	0.8	1.4	1.4	**3.0**	1.4	1.2
	8.7	**5.0**	1.4	0.3	1.0	1.2	0.6	0.4	1.1	1.0	**6.3**	0.9	1.1
	6.5	**3.1**	1.2	1.6	**5.3**	**6.4**	1.6	1.5	**2.3**	**9.0**	**14**	**3.0**	**3.2**
Mean SI	**6.6**	**4.5**	1.2	0.7	1.9	**2.2**	1.0	0.7	1.6	**3.4**	**6.5**	1.5	1.7
SE	1.9	1.1	0.2	0.5	1.9	2.3	0.5	0.5	0.5	3.5	4.7	0.8	0.9
Con-C	**7.1**	**6.1**	**2.1**	0.8	1.1	1.5	1.2	1.0	1.1	1.8	**3.1**	1.2	**2.0**
	7.8	**4.6**	1.3	0.4	1.1	**2.0**	0.6	0.5	0.8	1.2	**2.1**	1.1	**2.1**
	2.0	0.9	0.6	0.6	0.8	0.3	0.8	0.2	0.1	1.4	0.8	0.5	0.8
	9.1	**5.1**	1.1	1.0	0.8	**2.1**	1.1	0.8	1.6	2.1	**4.2**	**3.0**	1.1
Mean SI	**6.5**	**4.2**	1.3	0.7	1.0	1.5	0.9	0.6	0.9	1.6	**2.6**	1.5	1.5
SE	3.1	2.3	0.6	0.3	0.3	0.8	0.3	0.4	0.4	0.4	1.5	1.1	0.7
$MSP1_{19}$	1.2	1.0	**4.8**	1.0	1.0	1.6	1.1	0.6	1.2	1.6	1.8	1.0	1.1
	3.9	**2.1**	**9.1**	0.4	0.9	1.3	0.5	0.7	1.0	1.3	**3.0**	**2.8**	1.8
	1.8	1.8	1.8	1.1	1.1	0.6	1.1	0.9	0.9	1.0	1.0	1.6	0.6
	1.1	0.8	**3.2**	1.1	0.9	1.2	1.2	1.1	1.1	1.6	**2.0**	1.2	1.0
	0.3	0.7	0.6	0.3	1.1	0.5	0.7	0.4	0.5	0.9	0.4	0.4	0.8
Mean SI	1.7	1.3	**3.9**	0.8	1.0	1.0	0.9	0.7	0.9	1.3	1.6	1.4	1.1
SE	1.4	0.6	3.3	0.4	0.1	0.5	0.3	0.3	0.3	0.3	1.0	0.9	0.5

[a] Splenocytes from immunized C57BL/6 mice were cultured for 6 days with MSP2-A or $MSP1_{19}$ antigen (1 μg/ml) or peptide (10 μg/ml). Cultures were pulsed with [^{3}H]thymidine on day 5 and harvested on day 6.

[b] SI, stimulation indices. SI were determined as the geometric mean number of cpm of antigen-stimulated cells divided by the geometric mean number of cpm of medium control cells. Numbers of cpm for medium control cells ranged between 24 cpm and 274 cpm. Boldface values indicate SI values of ≥2. Peptide sequences were as follows: for C1, AENSAPTAEQTESPELQSAPC; for C2, TESPELQSAPENKGTGQHGHC; for C3, ENKGTGQHGHMHGSRNNHPQC; for C4, MHGSRNNHPQNTSDSQKECTC; for C5, NTSDSQKECTDGNKENCGAAC; for C6, DGNKENCGAATSLLNNSS; for C7, TSLLNNSSNIASINKFVVLIC; for C8, ASINKFVVLISATLVLSFAIC; and for L, FSPFLNFFMVKFSPFLNFFMVK.

though cells from some MSP2A-immunized mice also responded to the overlapping peptide, C7. Cells from all MSP2A-immunized mice and three of four Con-C-immunized mice proliferated in response to the C8 epitope. One mouse made responses to several peptides but also responded to an irrelevant peptide (L), suggesting that these responses were not induced by immunization.

Cytokine responses. Culture supernatants were collected 24 h, 3 days, or 5 days after in vitro restimulation of spleen cells from immunized mice with the original immunizing antigen and analyzed by multiplex bead array. At 24 h, all cytokine concentrations were below the detection limit of the assay (3.2 pg/ml), and these data are not shown.

Tumor necrosis factor alpha, IL-4, IL-3, and IL-1β could not be detected at any stage, indicating that cytokine concentrations were less than 3.2 pg/ml; IL-2, IL-5, granulocyte-macrophage colony-stimulating factor, and IFN-γ were detected on day 5, but concentrations did not differ between the groups of mice (data not shown). However, concentrations of IL-10 and IL-6 differed significantly between mice immunized with different antigens. Spleen cells from mice immunized three times with Con-C–GST and restimulated in vitro with Con-C–GST produced detectable levels of IL-6 and IL-10 within 3 days, and these concentrations had increased by day 5 (Fig. 3a and b). In contrast, spleen cells from mice immunized and restimulated with full-length MSP2-GST (MSP2A) produced barely detectable levels of IL-6 and IL-10 by day 3, and at day 5, concentrations were significantly lower than for Con-C. Cells from mice immunized and restimulated with $MSP1_{19}$-GST produced no detectable IL-6 or IL-10 at day 3, and levels remained extremely low at day 5; levels of both cytokines were significantly lower at day 5 than those induced by Con-C–GST.

To determine whether the cells producing IL-6 and IL-10 were specific for the C8 peptide, spleen cells from mice immunized with MSP2A, Con-C, or $MSP1_{19}$ fusion proteins were restimulated in vitro with peptide C8 and supernatants tested for IL-6 and IL-10. As expected, cells from mice immunized with $MSP1_{19}$-GST made extremely low levels of IL-6 and IL-10 when restimulated with the MSP2-derived C8 peptide, but cells from mice immunized with MSP2A-GST or Con-C–GST made significant levels of both IL-6 and IL-10 from day 3 onwards (Fig. 3c and d). Although concentrations of IL-6 and IL-10 induced by C8 were lower than those induced by recombinant antigen, they were significantly above background levels at day 5. Furthermore, cells from mice immunized with Con-C made higher levels of IL-6 and IL-10 than cells from mice immunized with MSP2A (Fig. 3a and b). These data indicate that while there may be more than one epitope within Con-C and MSP2A that induce high levels of IL-6 and IL-10 secretion from T cells, the C8 peptide represents such an epitope.

IL-10 and IFN-γ are required for class switching to IgG2b. To determine whether differences in levels of induction of IL-10 might explain the observed differences in IgG subclass ratios, antibody responses to MSP2A, Con-C, and $MSP1_{19}$ were analyzed in wild-type and IL-$10^{-/-}$ mice. Furthermore, although no differences were seen between the antigen constructs in their propensity to induce IFN-γ, as IFN-γ has previously been

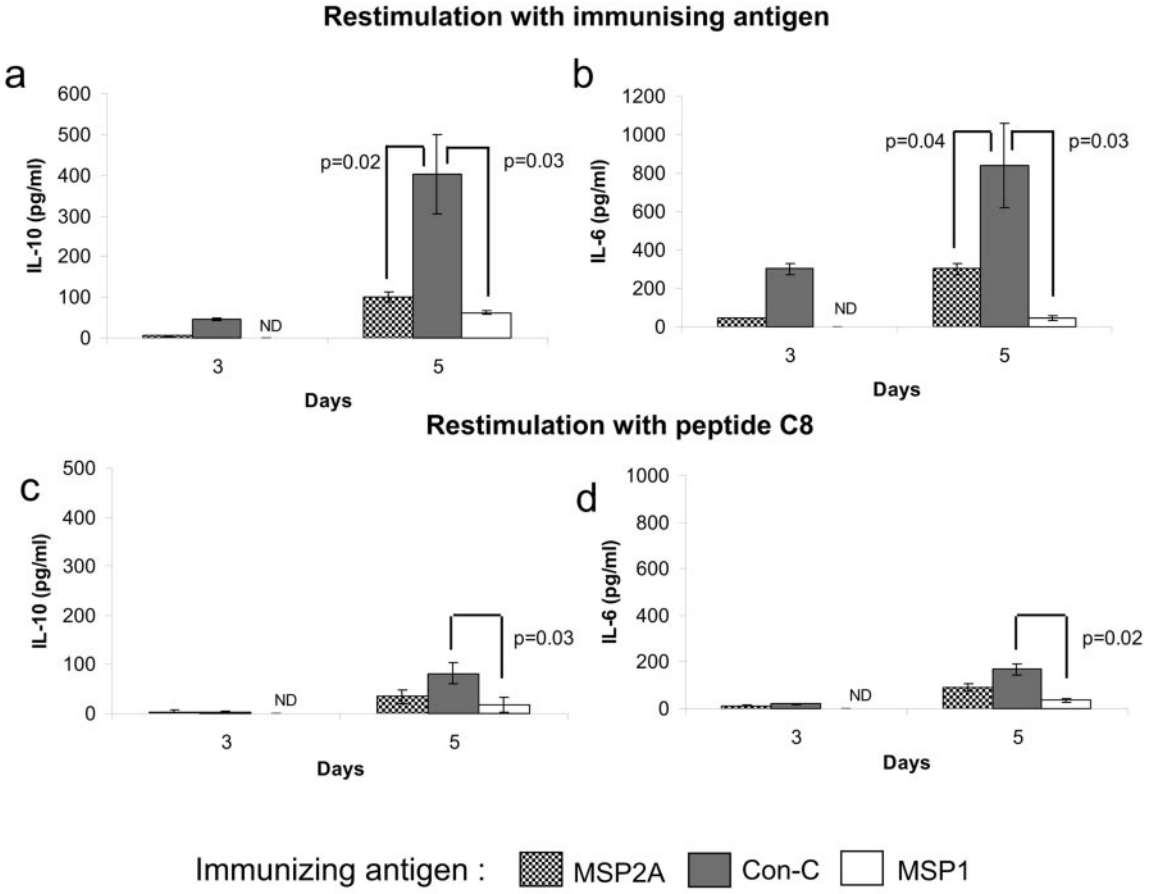

FIG. 3. Conserved C terminus of MSP2 induces rapid secretion of IL-6 and IL-10 by spleen cells from immunized C57BL/6 mice. Four weeks after the third immunization, spleen cells from mice (six per group) immunized with either MSP2A, Con-C, or $MSP1_{19}$ were collected and restimulated in vitro for 3 or 5 days with either the original immunizing antigen (a, b) or the C8 peptide (c, d). Culture supernatants were assayed by a cytokine bead assay. Data are presented as geometric mean cytokine concentrations (pg/ml). Cytokine values for cells cultured with GST alone (for a and b) or for cells cultured with an irrelevant peptide (for c and d) have been subtracted. ND, no detectable cytokine.

implicated in the regulation of class switching, we carried out a parallel series of immunizations in IFN-$\gamma^{-/-}$ mice.

Antibody titers were generally lower in IFN-$\gamma^{-/-}$ than in wild-type mice (Wilcoxon rank sum z, 2.44; $P < 0.01$), but for all the antigens studied, titers of IgG2b were more markedly reduced than titers of IgG1 (Fig. 4a); this effect was most marked for mice immunized with Con-C. Con-C-immunized wild-type mice developed a typical polarized IgG2b response, whereas in Con-C-immunized IFN-$\gamma^{-/-}$ mice, anti-Con-C antibodies were almost entirely IgG1.

In contrast to what was seen in IFN-$\gamma^{-/-}$ mice, total IgG titers were not significantly different between IL-$10^{-/-}$ and wild-type mice (Wilcoxon rank sum z, 1.23; $P = 0.217$) and IgG1 titers were actually higher in IL-$10^{-/-}$ mice (Fig. 4b). However, there was at least a 10-fold decrease in IgG2b titers in IL-$10^{-/-}$ mice in response to all three antigens. Thus, both IFN-γ and IL-10 seem to be required for optimal class switching to IgG2b.

The conserved C terminus of MSP2 is not a major target for antibodies. Having putatively identified an epitope (C8) in the conserved C terminus of MSP2 that might be implicated in T-cell-mediated class switching to IgG2b, we wanted to know whether C8 might interact directly with C8-specific B cells to influence the class switch. To determine whether C8 represents a significant B-cell epitope, sera from mice immunized with Con-C–GST or MSP2A-GST were screened by ELISA for binding to the panel of C-terminal peptides. The Con-C–GST immune sera failed to show any significant binding (defined as an OD greater than or equal to the OD of binding to the irrelevant peptide, L) to any of the Con-C peptides (data not shown). The MSP2A-GST immune sera showed low-titer binding to C3 (midpoint titer, 986 ± 175 [mean ± standard deviation]), C5 (midpoint titer, 2,537 ± 348), and C6 (midpoint titer, 394 ± 123) but not to C8 (titer below that of the irrelevant peptide).

The conserved C terminus of MSP2 overcomes the effects of adjuvants to drive IgG2b class switching to linked proteins. The observation that Con-C drives class switching to IgG2b, even in the presence of an adjuvant that normally induces IgG1 responses, indicates that this epitope might be able to override the normal class-switching mechanisms of linked proteins and might thus be incorporated into chimeric proteins to induce IgG2b antibodies to unrelated antigens. To test this hypothesis, we compared the subclasses of the antibody response to an unrelated protein (the fusion protein partner, GST) when it was used as an immunogen on its own and when it was coupled to Con-C, $MSP1_{19}$, or MSP2A. To determine whether the C8

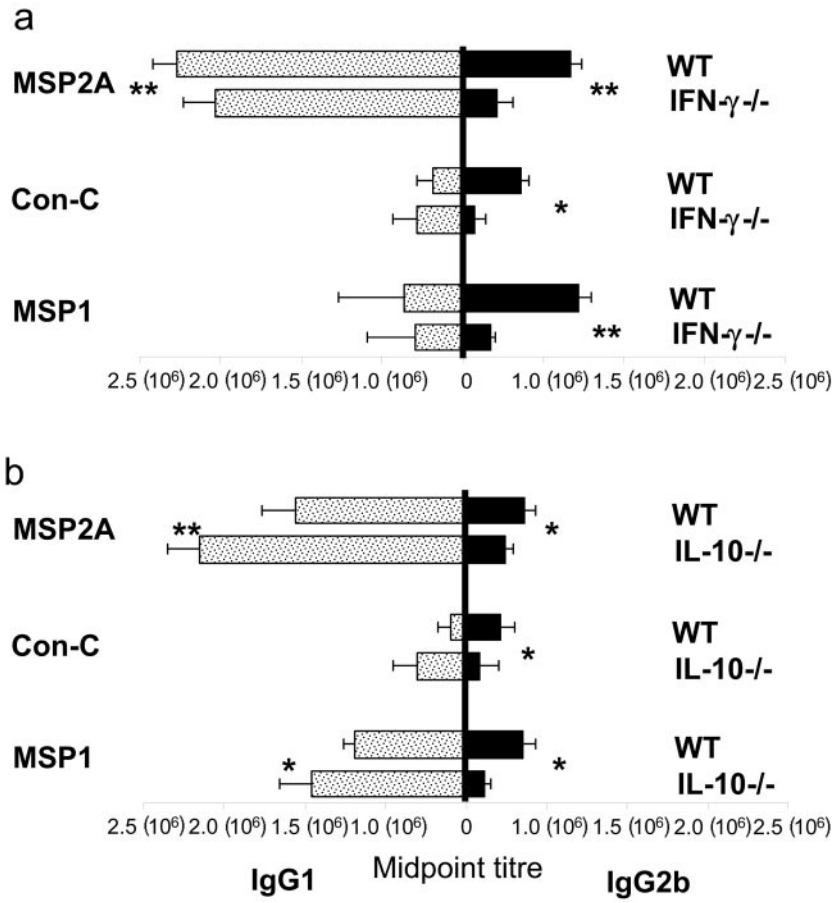

FIG. 4. B cells from IFN-γ-deficient and IL-10-deficient mice fail to class switch to IgG2b. Wild-type (WT), IFN-$\gamma^{-/-}$ (a), and IL-$10^{-/-}$ (b) mice (six per group) were immunized with $MSP1_{19}$, MSP2A, or Con-C, and median midpoint titers of IgG1 and IgG2b were measured by ELISA 2 weeks after the final immunization. The statistical significance of differences between wild-type and cytokine-deficient mice was determined using nonparametric (Wilcoxon signed rank) tests and is indicated. *, $P < 0.05$; **, $P < 0.005$.

epitope of Con-C is the crucial T-cell epitope driving the IgG2b response, we also immunized mice with chimeric proteins comprising the C8 peptide conjugated to $MSP1_{19}$-GST (C8-$MSP1_{19}$-GST) and (as a control) with $MSP1_{19}$-GST conjugated to an irrelevant peptide, L (L-$MSP1_{19}$-GST) and characterized the antibody responses to both the $MSP1_{19}$ and GST components of the chimeric immunogens. Finally, to rule out any covert effect of the inclusion of GST in the recombinant proteins, we immunized mice with BSA conjugated to C8 or to the control L peptide.

The conserved C terminus of MSP2 drives class switching to IgG2b when MSP2 is linked to a non-MSP2 protein. As shown in Fig. 2b, GST alone induces IgG1 antibodies. When mice were immunized with GST fused to $MSP1_{19}$, the anti-GST antibodies were almost entirely IgG1 (Fig. 5a); some anti-GST IgG2b was induced, but titers were fivefold lower than for IgG1. Similarly, when mice were immunized with GST fused to full-length MSP2 (MSP2A-GST), anti-GST antibodies were entirely of the IgG1 subclass (Fig. 5b), although after the third immunization, the anti-GST titers were approximately 10-fold lower than those induced by GST-$MSP1_{19}$. In complete contrast, mice immunized with GST–Con-C made anti-GST antibodies that were entirely of the IgG2b subclass (Fig. 5c), with titers for other subclasses being below background levels.

Peptide C8 from MSP2 drives class switching to IgG2b to linked malarial and nonmalarial proteins. IgG1 and IgG2b titers to $MSP1_{19}$-GST and to GST alone were compared among mice immunized with $MSP1_{19}$-GST, L-$MSP1_{19}$-GST, or C8-$MSP1_{19}$-GST, and the IgG2b/IgG1 ratio was calculated (Fig. 6). In accordance with previous experiments, mice immunized with $MSP1_{19}$-GST made strong IgG1 responses to $MSP1_{19}$ after three immunizations and made little or no IgG2b; IgG2b/IgG1 ratios were thus less than 1 (Fig. 6a). Conjugating the irrelevant 20-amino-acid peptide (L) to $MSP1_{19}$-GST made no appreciable difference to the anti-$MSP1_{19}$ response. By contrast, conjugation of the C8 peptide to $MSP1_{19}$-GST led to a significant increase in IgG2b antibody responses to $MSP1_{19}$-GST, which was detectable after two immunizations (data not shown) and which increased further after three immunizations such that IgG2b/IgG1 ratios were now significantly above one in three out of four immunized mice (Fig. 6a). Furthermore, absolute IgG2b titers were significantly higher in C8-$MSP1_{19}$-GST-immunized mice than in L-$MSP1_{19}$-GST-immunized mice after both two (Wilcoxon rank sum z, -8.57; $P < 0.001$) and three (Wilcoxon rank sum z, -10.82; $P < 0.001$) immunizations.

Essentially similar results were seen for anti-GST and anti-BSA responses, with mean IgG2b/IgG1 ratios being less than 1

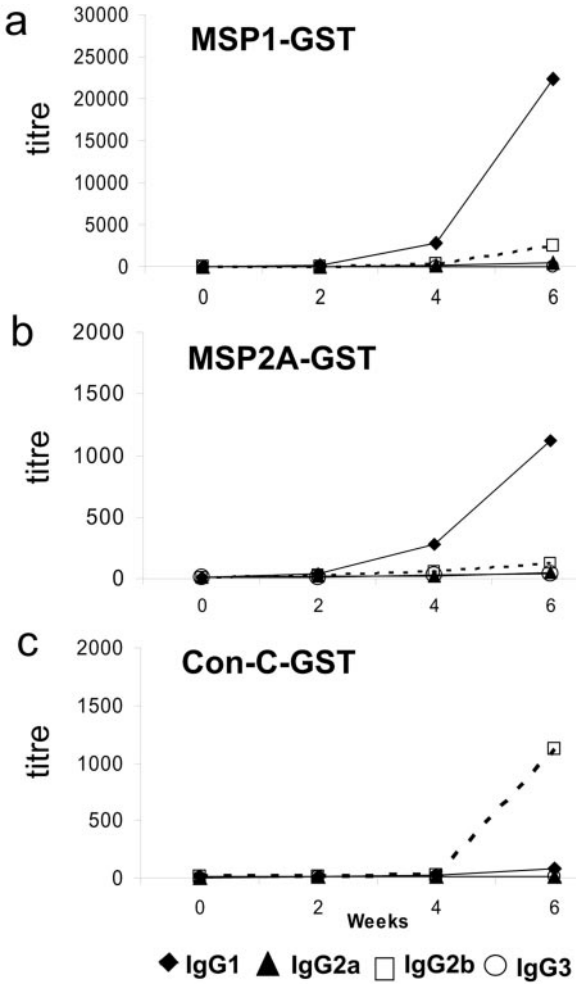

FIG. 5. The C8 peptide of MSP2 drives class switching of the anti-GST antibody response to IgG2b. Mice (four per group) immunized with (a) $MSP1_{19}$-GST, (b) MSP2A-GST, or (c) Con-C–GST were tested for antibodies to GST alone. Data represent median midpoint titers of each IgG subclass.

in mice immunized with protein alone or protein conjugated to peptide L but above 1 in mice immunized with protein conjugated to peptide C8 (Fig. 6b and c); differences in IgG2b/IgG1 ratios were statistically significant between L-conjugated and C8-conjugated proteins for both GST (Wilcoxon rank sum z, -3.26; $P < 0.001$) and BSA (Wilcoxon rank sum z, -2.44; $P < 0.01$).

DISCUSSION

The objective of this study was to explore the mechanisms underlying IgG subclass switching during immunization with defined antigens, in this case candidate malaria vaccine antigens, with the long-term aim of being able to engineer synthetic vaccines that preferentially induce high titers of protective antibody subclasses. This is one of very few studies that have identified antigen-specific stimuli for selective Ig class switching in B cells, and this study is, we believe, the first to demonstrate cytokine-dependent antigen-mediated class switching to IgG2b.

The genes encoding the conserved domains of the IgG heavy chains of humans and mice diversified after speciation, and it is not possible to identify clear homologues of the different IgG subclasses in the two species. However, parallels can be drawn in terms of their function and regulation. In particular, human IgG3 shares many features with mouse IgG2b. Both human IgG3 and mouse IgG2b are minor components of normal serum, bind with high affinity to Fc receptor, fix complement, are preferentially induced during Th1 immune responses, and are especially effective at mediating immunity to blood stage malaria infection (1, 8, 18, 23, 48, 50, 51). These observations, together with accumulating evidence that the machinery for class switch recombination is conserved between humans and mice (27), suggest that there may also be conservation of the signals required for initiation of specific Ig isotype and subclass switching and thus that information derived from mouse models may be directly applicable to studies of human B cells.

We have capitalized on the ability of the *P. falciparum* MSP2 protein to spontaneously induce a polarized IgG3 response in humans and have shown that fragments of this protein induce equally polarized IgG2b responses in C57BL/6 mice. We have also shown that a conserved 20-amino-acid peptide (C8) from the extreme C terminus of MSP2 encodes a T-cell epitope that preferentially induces switching to IgG2b in B cells responding to linked epitopes. IgG2b class switching seems to be linked to rapid induction of T cells to produce IL-10 and, possibly, IL-6 in addition to IFN-γ. The effect of this T-cell epitope overrides the effect of a strong adjuvant that has been shown to preferentially induce IgG1 and IgG2a antibodies (58), overrides any effect of the carrier GST molecule, and can alter the pattern of IgG responses to non-MSP2 antigens to which it is linked.

Our data suggest that conjugation of MSP2 sequences to other malaria proteins in a chimeric vaccine might enhance IgG3 antibody responses in humans and, given the large body of evidence that IgG3 antibodies are particularly effective at mediating opsonization and phagocytosis of parasitized erythrocytes (3, 4, 26), that this may result in enhanced protection. The precise elements of MSP2 that induce preferential class switching in humans need to be identified, and there is no guarantee that they will map to the Con-C region. Indeed, Con-C does not induce a preferential IgG2b switch in BALB/c mice (data not shown), suggesting that the minimal epitope within C8 that is presented to IgG2b-inducing T cells by $H\text{-}2^b$ in C57BL/6 mice cannot be presented in the context of $H\text{-}2^d$. The C8 peptide itself is not present in the mature MSP2 molecule, as it lies within the region of the C terminus that is removed posttranslationally in order to allow attachment of the glycosylphosphatidylinositol anchor, and thus is not expressed at the merozoite surface. Nevertheless, it seems likely that the peptide is retained long enough and in sufficient amounts to function as a T-cell epitope during natural malaria infections since proliferative T-cell responses to sequences within C8 have been reported in malaria-exposed individuals from the Solomon Islands (41).

Skewing of the MSP2 antibody response to IgG3 is an almost universal phenomenon in malaria-exposed humans, indicating that MSP2 contains either numerous different IgG3-inducing T-cell epitopes or, perhaps more likely, a few such epitopes with promiscuous HLA-DR binding specificities; several malaria epitopes with broad HLA-DR binding properties have been described previously (2, 17, 22, 31), and MSP2 T-cell

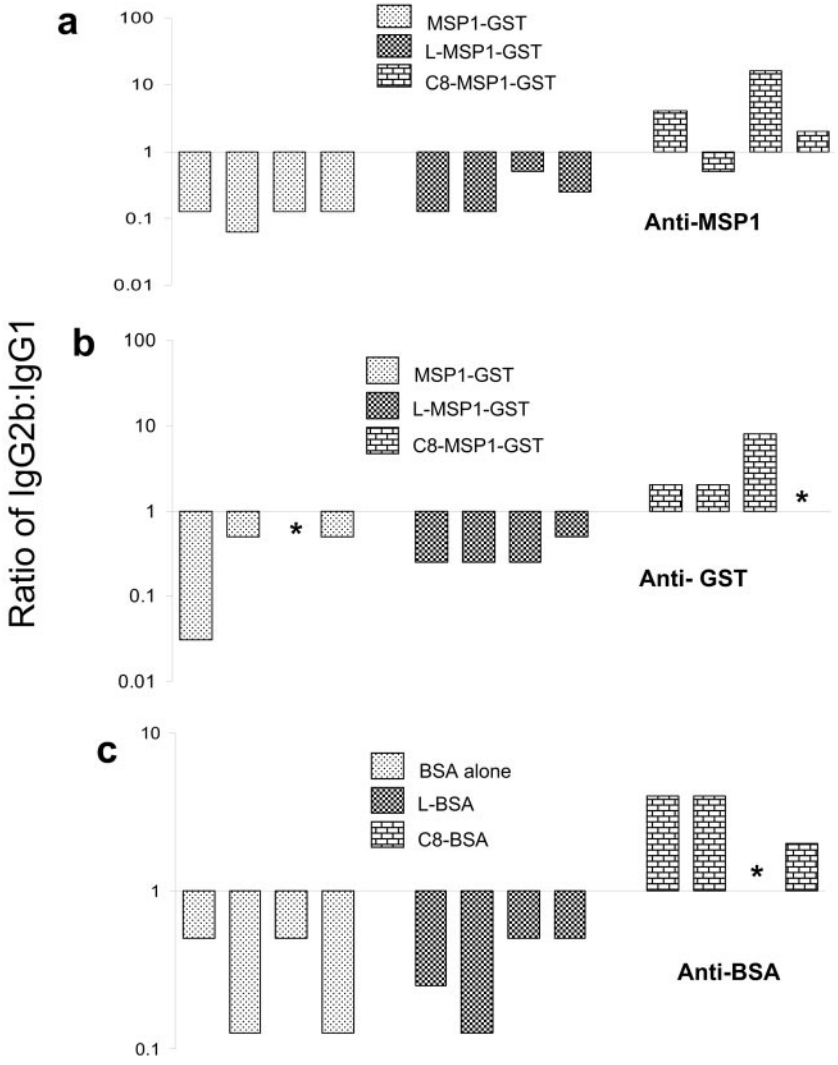

FIG. 6. Peptide C8 drives class switching of the antibody response to $MSP1_{19}$, GST, and BSA to IgG2b. Ratios of midpoint titers of IgG2b to IgG1 antibodies to (a) $MSP11_{19}$ (b) GST, and (c) BSA in the sera of C57BL/6 mice (four per group) immunized with (a, b) $MSP1_{19}$-GST, $MSP1_{19}$-GST conjugated with peptide C8 (C8-$MSP1_{19}$-GST), $MSP1_{19}$-GST conjugated with an irrelevant peptide (L-$MS1_{19}$-GST), (c) BSA alone, BSA conjugated to peptide L (L-BSA), or BSA conjugated to C8 (C8-BSA). Each bar represents the IgG2b/IgG1 ratio for serum from one mouse. * indicates a serum where the ratio was 1.0.

epitopes with broad recognition within an outbred human population have been described previously (53). The demonstration here that the epitopes of interest are likely to induce the production of high levels of IL-6 and IL-10, combined with the use of algorithms for identifying promiscuous HLA-DR binding epitopes (16), will facilitate the search for such epitopes.

As the skewing of human anti-MSP2 responses to IgG3 is seen for multiple members of both MSP2 serogroups (42, 52), we hypothesized that, if factors within the MSP2 sequence itself are responsible for IgG3 polarization, they are most likely to lie within the conserved N- or C-terminal sequences. We also hypothesized that the crucial interactions for class switching were more likely to take place between MSP2 and T cells than between MSP2 and B cells, as following natural infection, human antibodies to MSP2 recognize predominantly dimorphic and polymorphic sequences, with little or no antibody to the conserved sequences being detectable (52), suggesting that there are no dominant B-cell epitopes within the highly conserved regions of the molecule. Furthermore, although conserved or cross-reactive epitopes for antibodies are present throughout the MSP2 molecule (24), dominant epitopes for human (41, 53) and murine (40) T cells have been identified within the conserved sequences of MSP2. The data presented here essentially support these predictions in that (i) mice immunized with full-length MSP2 made little or no antibody to the conserved C terminus, (ii) we have identified a dominant T-cell epitope within the conserved C terminus of the molecule which is able to drive class switching to IgG2b, and (iii) this peptide is not, in itself, a major target for anti-MSP2 antibodies.

Clearly, T-cell epitopes within the conserved C terminus are not the only MSP2 epitopes that drive IgG2b class switching in C57BL/6 mice, as dimorphic and polymorphic MSP2 proteins were also able to induce significant IgG2b responses; however, these responses became evident only after two or three immu-

nizations, suggesting that these IgG2b-inducing T-cell epitopes may be subdominant. This gradual induction of IgG2b antibodies is, in itself, unusual, as repeated immunization of mice with soluble protein has a very strong tendency to polarize antibody production towards IgG1 (29). Interestingly, even though they contained the conserved C terminus, the full-length MSP2 proteins were the least able to induce strong IgG2b responses, suggesting that other T-cell epitopes may assume immunodominance in the context of the full-length protein. It is possible that the expression of these full-length proteins as hexa-His fusions, rather than as GST fusions, may have influenced the class switch response, in which case the choice of fusion partner for recombinant protein vaccines may be an important parameter to consider when attempting to optimize Ig subclass responses. Nevertheless, antibody titers to these hexa-His proteins still increased after three immunizations, and it is likely that IgG2b would eventually come to dominate the response to the full-length proteins also. This is reminiscent of what is seen in human populations with the switch from MSP2-specific IgG1 to IgG3 taking place over time, with increasing age and increasing malaria exposure, in areas of high endemicity (23a, 51) but not in areas of hypoendemic malaria transmission (54).

In addition to its potential applications for improving qualitative aspects of the antimalarial antibody response, our study also provides entirely novel data on factors that control class switching to IgG2b in murine B cells. Many of the cytokine signals for class switching appear to be highly conserved between mice and humans; for example, IL-5 induces DNA rearrangement, while IL-4, transforming growth factor β (TGF-β), and IFN-γ mediate the transcription of C_H genes for IgG1, IgA, and IgG2a, respectively, in mice, and their equivalents (IgG4, IgA, and IgG1) in humans (49). Our data suggest that regulation of class switching to murine IgG2b and human IgG3 may also be very similar and that the ability of murine T cells to preferentially induce a class switch to IgG2b is linked to their ability to rapidly and selectively secrete large amounts of IL-10 and/or IL-6 in addition to IFN-γ. As such, this study represents the first demonstration of a T-cell-dependent, antigen-specific induction of IgG2b. Previously, a T-cell-independent antigen, bacterial lipopolysaccharide (but not dextran-conjugated anti-IgD), plus TGF-β have been shown to drive the switch to IgG2b in BALB/c mice (34); however, in human surface IgD^+ B cells, secretion of IgG3 can be induced by IL-10 (6), which fits well with the data presented here. Furthermore, in human schistosomiasis, IL-10 and IFN-γ responses have been linked to IgG3 production (39), which accords well with the data presented here from IL-10-deficient and IFN-γ-deficient mice. One explanation for the role of these two cytokines that accords with all these findings might be that IFN-γ suppresses transcription of γ 1 and ε genes (13, 28, 33) but that IL-10 and/or TGF-β actively promote switching to γ 2b (in mice) or γ 3 (in humans). The role of IL-6 in class switching to IgG2b is less well defined and clearly deserves further investigation.

In summary, this study demonstrates that chimeric vaccine antigens, incorporating T-cell epitopes that promote IgG switching to specific subclasses, can be used to modify the antibody response to large recombinant proteins even in the context of a strong adjuvant. Furthermore, this study represents the first demonstration of antigen-driven, T-cell-dependent class switching to IgG2b in murine B cells and strongly implicates IL-10, IFN-γ, and, possibly, IL-6 in this process. Experiments are under way to further elucidate the cytokine requirements for T-cell-dependent, antigen-specific IgG2b class switching in murine B cells.

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This study was approved by the Animal Ethical Review Committee of the London School of Hygiene and Tropical Medicine.

No conflicts of interest need to be declared.

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