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Cutting Edge: The IgG Response to the Circumsporozoite Protein Is MHC Class II-Dependent and CD1d-Independent: Exploring the Role of GPIs in NK T Cell Activation and Antimalarial Responses[1]

Alberto Molano,* Se-Ho Park,† Ya-Hui Chiu,† Sandy Nosseir,* Albert Bendelac,† and Moriya Tsuji[2]*

Biochemical analysis has suggested that self GPI anchors are the main natural ligand associated with mouse CD1d molecules. A recent study reported that Vα14$^+$ NK T cells responded to self as well as foreign (parasite-derived) GPIs in a CD1d-dependent manner. It further reported that the IgG response to the *Plasmodium berghei* malarial circumsporozoite (CS) protein was severely impaired in CD1d-deficient mice, leading to a model whereby NK T cells, upon recognition of CD1d molecules presenting the CS-derived GPI anchor, provide help for B cells secreting anti-CS Abs. We tested this model by comparing the anti-CS Ab responses of wild-type, CD1d-deficient, and MHC class II-deficient mice. We found that the IgG response to the CS protein was solely MHC class II-dependent. Furthermore, by measuring the response of a broad panel of CD1d-autoreactive T cells to GPI-deficient CD1d-expressing cells, we found that GPIs were not required for autoreactive responses. ***The Journal of Immunology*****, 2000, 164: 5005–5009.**

Recent studies support the notion that CD1 molecules form part of a singular Ag-presenting system specializing in the presentation of lipids and glycolipids for T cell recognition (1). Group I CD1 molecules, such as human CD1b and CD1c, can bind and present lipid Ags present in mycobacteria such as lipoarabinomannan (2), mycolic acids (3), and glucose monomycolate (4) for recognition by phenotypically diverse T cell lines or clones. Group II CD1 molecules such as murine and human CD1d are known to present synthetic glycolipids such as α-galactosylceramide (5, 6) to NK T cells, resulting in potent cellular activation. However, this compound has only been found in marine sponges, so there has been great interest in identifying naturally presented ligands capable of activating NK T cells within a more physiological context.

NK T cells (7) are the predominant T cells known to be associated with CD1d-restricted recognition. These CD4$^+$ or double-negative cells are characterized by the expression of an invariant TCR α-chain (Vα14-Jα281) and a limited TCR β-chain repertoire, together with the unusual coexpression of cell-surface markers commonly associated with NK cells. Upon TCR engagement, NK T cells secrete not only large amounts of IL-4, IL-5, and IL-10, but also IFN-γ and TNF-β, cytokines which have opposite effects on Th1/Th2 differentiation. This has generated some controversy regarding their specific influence in CD4$^+$ Th cell differentiation and on how the subsequent adaptive immune response may be biased. Some studies implicate them in Th2 responses (8), whereas others associate them with the inhibition of Th2 responses (9) or with the generation of Th1 responses (10).

Efforts to identify ligands naturally associated with CD1d have relied on a variety of biochemical approaches. Joyce et al. (11) found self GPI anchors to be the main natural ligand associated with mouse CD1d molecules. Using various purified or synthetic GPIs, Schofield et al. (12) subsequently showed that Vα14$^+$ NK T cells were stimulated by self as well as foreign (parasite-derived) GPIs in a CD1d-dependent manner and that GPI recognition was mediated by the glycan moiety, in that the phosphatidylinositol (PI)[3] core by itself was not stimulatory. In support of these findings, these authors also reported that the IgG response to the *P. berghei* malarial circumsporozoite (CS) Ag, which is believed to be GPI-anchored (13), was severely reduced in CD1d-deficient mice. This would indicate that most T cell help for the production of anti-CS IgG was provided by NK T cell recognition of CD1d/GPI complexes on the surface of CS-specific B cells. A conclusion from all these studies is that Vα14$^+$ NK T cells recognize CD1d complexed with self or foreign GPIs, and that this specific recognition is linked to their regulatory roles in autoimmune diseases as

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[3] Abbreviations used in this paper: PI, phosphatidylinositol; CS, circumsporozoite; IFA, immunofluorescence assay.

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well as in a variety of infectious conditions. In this study, we measured the response of a broad panel of CD1d-autoreactive T cells to wild-type or GPI-deficient (deficient in the PIG-A enzyme required for GlcNac-PI synthesis) CD1d-expressing cells. Contradicting our initial predictions, we found that CD1d-autoreactive T cells were unaffected by the dramatic alteration in GPI structure produced by the PIG-A deficiency. In addition, we compared the anti-CS Ab response of wild-type and CD1d-deficient mice (of both the B6 and BALB/c genetic backgrounds) after direct i.v. inoculation of irradiated sporozoites or after exposure to infected mosquito bites. Our results do not support a role for CD1d-mediated activation of Vα14$^+$ NK T cells as providers of cognate help for IgG anti-CS responses. In contrast, a comparison of the anti-CS Ab response of wild-type and MHC class II-deficient mice showed this response to be solely MHC class II-dependent. This is in line with a previous study that identified multiple Th cell epitopes in the *P. berghei* CS protein (14).

Materials and Methods

CD1d1-transfected GPI mutant lines

Matched wild-type and Class A mutants of GPI biosynthesis were obtained from Dr. Hyman (Salk Institute) for two tumor lines, BW5147 and S49. The class A mutants (termed BW-Thy-1-a and S49-Thy-1-a) have a block in the transfer of *N*-acetylglucosamine to the PI acceptor (15). All four cell lines, which constitutively express very low levels of CD1d, were stably transfected with CD1d1 using plasmid pCD113 as described (16) and selected by G418 treatment and multiple cell sortings to ensure stable expression of comparable levels of CD1d1.

In vitro stimulation of CD1d-autoreactive T cells

T cells were cultured for 18 h in the presence of CD1d transfectants (5×10^4 responders and 5×10^4 transfectants) in 100 μl of a 1:1 mixture of Click's medium and RPMI 1640 (Biofluids, Rockville, MD) enriched with 10% heat-inactivated FCS, glutamine, antibiotics, and 5×10^{-5} 2-ME. IL-2 or IL-4 released in the supernatant was measured using the CTLL or CT-4S bioassays, respectively, as described (17). Fresh T cell responders were thymocytes from Vα14-Jα281 TCRα transgenic mice (18) recovered after 5 days in culture with 2.25 μg/ml Con A and IL-4 (10 ng/ml), washed, and stimulated as indicated above to measure IL-4 secretion. CD1d-autoreactive hybridomas have been described previously (16, 17, 19) and were stimulated as described above to measure IL-2 release.

Mice

C57BL/6 and BALB/c mice were obtained from the National Cancer Institute (Bethesda, MD). CD1-deficient (CD1D1-null) mice, which do not have NK T cells and have no residual expression of CD1 on B cells (8, 20), were used after six to nine backcrosses onto the C57BL/6 and BALB/c backgrounds. MHC class II-deficient mice (I-A$_\beta^b$$-/-$) (21) were used after six backcrosses onto the C57BL/6 background. Eight- to 10-wk-old mice of both sexes were used. All mice were housed in autoclaved cages and were given autoclaved food and water.

Immunizations

P. berghei (NK65 strain) was maintained as described (22). Anesthetized mice were subjected to the bites of gamma-irradiated (15,000 rad; 1 rad = 0.01 Gy) malaria-infected *Anopheles stephensi* mosquitoes for 10 min/day for 4 (BALB/c background, CD1-deficient mice) or 8 (C57BL/6 background, CD1-deficient mice) days, rested for 10 days, and boosted for 4 more days. For i.v. immunizations, mice were injected in the tail vein with 10^5 dissected, irradiated salivary gland sporozoites and boosted 2 wk later with the same number of irradiated sporozoites. Sera from the immunized mice were collected 12 days after the last immunization.

ELISA and indirect immunofluorescence assay (IFA)

IgG Ab titers directed against the *P. berghei* CS protein were determined by coating Immulon-2 plates (Dynatech Laboratories, Alexandria, VA) overnight at 4°C with 0.5 μg/ml of the B8 multiple Ag peptide in PBS. The B8 multiple Ag peptide, described in detail before (23), contains eight copies of the immunodominant epitope $(DPPPPNPN)_2G$ of the CS protein of *P. berghei*. After washing three times with PBS/0.05% Tween 20, plates were blocked for 1 h at 37°C with PBS/3% BSA. Plates were incubated for 1 h with the serum dilutions. After extensive washing, peroxidase-conjugated goat affinity-purified anti-mouse IgG Fc (Cappel, Aurora, Ohio) was added. Plates were developed by adding the substrate 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonate) (Kirkegaard & Perry, Gaithersburg, MD). End-titers were defined as the last serum dilution (titration) giving values statistically different from those of the preimmune sera. A serum titer of 1/100 was the background cutoff. IFA was performed as described (24).

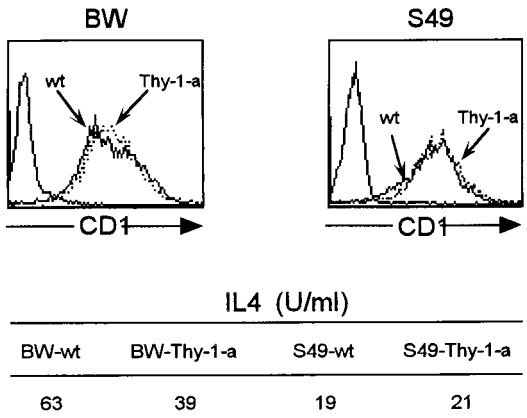

FIGURE 1. Vα14 transgenic cells respond similarly to CD1d expressed by wild-type or GPI-deficient cell lines. Comparable levels of CD1 expression are found on stably transfected GPI-deficient (Thy-1-a) as well as wild-type (wt) tumor lines BW and S49. The figure also shows the response of Vα14 transgenic cells to these transfectants, measured as IL-4 released into the supernatant (IL-4 release by Vα14 transgenic cells alone or in the presence of untransfected tumor lines was <2 U/ml). Similar results were obtained in six experiments using wild-type and GPI-deficient CD1d transfectants matched for comparable expression of CD1d1.

Results

Vα14 transgenic cells respond similarly to CD1d expressed by wild-type or GPI-deficient cell lines

PIG-A mutants (15) have a block in the transfer of *N*-acetylglucosamine to the PI acceptor. To examine the effects of this mutation on CD1d autoreactivity, we stably transfected BW-Thy-1-a and S49-Thy-1-a mutants, as well as the parental tumor lines BW 5147 and S49, with CD1d1. Fig. 1 shows that comparable levels of CD1d expression were present in the wild-type and mutant cell lines used for these experiments. It also shows that the responses of Vα14 transgenic cells (18) to these transfectants, measured as IL-4 released into the supernatant, are similar. It is important to emphasize that the Vα14 transgenic cells constitute a polyclonal population of cells expressing the invariant TCR α-chain associated with a broad range of endogenous TCR β-chains (18, 19). Therefore, the results reflect the overall response pattern of a broad range of Vα14-based TCRs.

CD1d-autoreactive hybridomas respond similarly to CD1d expressed by wild-type or GPI-deficient cell lines

We also examined the response of individual, CD1d-autoreactive hybridomas to CD1d expressed by wild-type or GPI-deficient cell lines, including one Vα14-Jα281/Vβ8 hybridoma (DN32D3) as well as 13 non-Vα14, CD1d-autoreactive hybridomas (Fig. 2). Although there is some level of variation from experiment to experiment, overall the data conclusively demonstrate that GPI expression is not required for recognition by a broad panel of CD1d-autoreactive T cells belonging to both the Vα14-positive and Vα14-negative subsets.

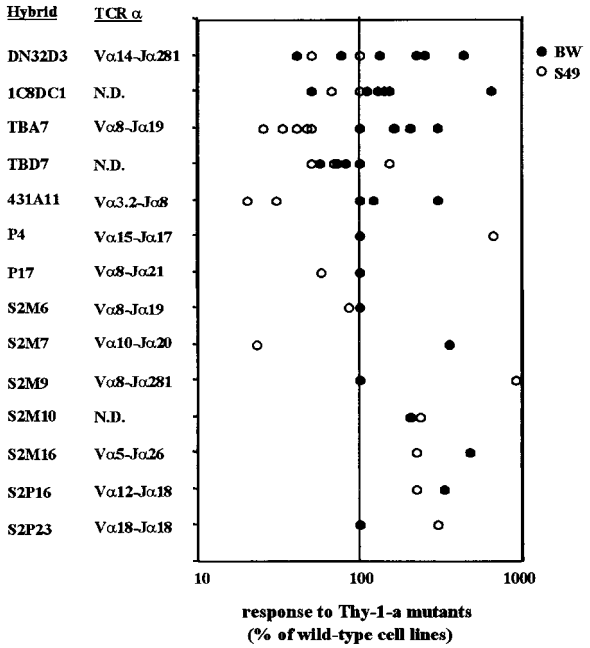

FIGURE 2. CD1d-autoreactive hybridomas respond similarly to CD1d expressed by wild-type or GPI-deficient cell lines. Each circle represents a separate experiment in which the response (IL-2 release) of an individual hybridoma to GPI-deficient, CD1d-transfected BW (●) or S49 (○) is expressed as a percentage of the response to wild-type CD1d-transfected BW and S49. Tested hybridomas include one Vα14-Jα281/Vβ8 (DN32D3) as well as 13 non-Vα14-autoreactive hybridomas.

The absence of CD1d expression in knockout mice does not significantly influence the IgG anti-CS response

To examine the effects of CD1d on the induction of IgG anti-CS Abs, we exposed groups of CD1-deficient (−/−), CD1 heterozygous (+/−), and wild-type (+/+) mice, in parallel, to the bites of irradiated mosquitoes infected with *P. berghei*. The experiments were conducted using CD1-deficient mice of the C57BL/6 (Fig. 3*A*) and BALB/c (Fig. 3*B*) backgrounds. Sera were collected 12 days after the final boosting, and IgG anti-CS titers were determined by ELISA. No significant difference was observed between any of the groups. Identical results were obtained by IFA using sporozoites as an Ag (not shown). The experiments were also performed after direct i.v. inoculation of irradiated sporozoites in CD1-deficient mice of the C57BL/6 background (Fig. 4). Again, no significant differences were observed. We conclude that the absence of CD1d expression in knockout mice does not significantly influence the titers of IgG directed against the CS protein of *P. berghei*.

The IgG anti-CS response is solely MHC class II-dependent

If the IgG response to the CS protein of *P. berghei* is not dependent on CD1d, then it should be predominantly MHC class II-dependent. We tested this hypothesis by inoculating irradiated sporozoites into wild-type, CD1-deficient, and MHC class II-deficient mice, all of the C57BL/6 background. As shown in Fig. 4, whereas the mean titers for the wild-type (1/4400) and the CD1-deficient (1/4800) groups are comparable, the IgG anti-CS titers of the MHC class II-deficient mice were undetectable (less than 1/100). This result indicates that the IgG anti-CS response is entirely dependent on MHC class II molecules.

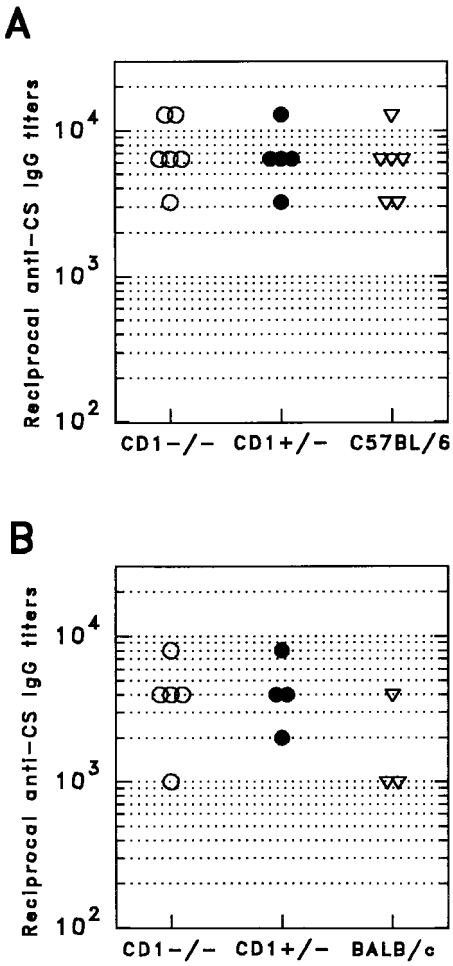

FIGURE 3. The IgG response to the CS protein is CD1d-independent. *A*, Groups of CD1-deficient ($CD1^{-/-}$), CD1-heterozygous ($CD1^{+/-}$), or wild-type C57BL/6 mice were repeatedly exposed to the bites of irradiated mosquitoes infected with *P. berghei*. The figure shows IgG titers directed against the immunodominant repeat sequence of the *P. berghei* CS protein. *B*, Same as in *A* but using mice of the BALB/c background.

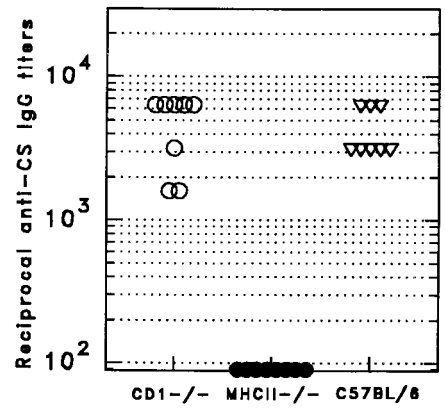

FIGURE 4. The IgG response to the CS protein is solely MHC class II-dependent. Groups of MHC class II-deficient ($MHCII^{-/-}$), CD1-deficient ($CD1^{-/-}$), or wild-type C57BL/6 mice were primed and boosted i.v. with 10^5 irradiated *P. berghei* sporozoites. The figure shows IgG Ab titers directed against the immunodominant repeat sequence of the *P. berghei* CS protein.

Discussion

The function of NK T cells in the immune response as well as a precise identification of the natural CD1d-presented Ags responsible for their in vivo activation remain elusive. Studies of CD1-reactive T cells that display autoreactivity, i.e., a response without any foreign Ag added, have raised the issue of what exactly is being recognized by these cells (16). We have described two main subsets of CD1d-autoreactive cells based on the expression of semiinvariant Vα14-Jα281/Vβ8 or variable, non-Vα14-based TCR. These two subsets differ in their requirement for the tyrosine-based endosomal sorting motif encoded in the cytoplasmic tail of CD1, suggesting that they recognize distinct families of self Ags loaded in different cellular compartments (17). The crystal structure of CD1d revealed a very hydrophobic interior groove with two deep, large pockets, occupied by discontinuous electron density (25), apparently an acyl chain-containing ligand (26), and biochemical analysis (11) identified cellular GPI as a major natural ligand of CD1d1. Recently, Naidenko et al. (26) demonstrated binding of a biotinylated phospholipid to human CD1d by surface plasmon resonance. Although the recognition of "empty" or lipid-stabilized CD1d molecules by autoreactive T cells is a formal possibility, the studies mentioned above suggest that self GPIs could be critical ligands that are intimately involved in the activation of these autoreactive T cells. In accordance with these findings, Schofield et al. (12) reported that various self or foreign parasite-derived GPIs were capable of activating and expanding Vα14$^+$ NK T cells in the spleen of immunized mice. This reactivity was found to be MHC class II-independent and CD1-restricted, and the glycan moiety was found to be essential because the PI core by itself was not stimulatory. All these studies suggest that self and foreign GPIs are critical determinants of Vα14$^+$ NK T cell reactivity and that their specific recognition must be somehow linked to immunoregulatory roles. Consequently, altering the structure of cellular GPIs should have a significant effect on the autoreactivity of Vα14$^+$ NK T cells. However, we found that both Vα14 transgenic cells and CD1d-autoreactive hybridomas responded similarly to wild-type and GPI-deficient cell lines. Although it remains possible that some CD1d-autoreactive cells might recognize the PI core without the glycan moiety (which persists in PIG-A mutants; Ref. 27) or that they react to other non-GPI glycolipids, our results raise questions about the claim that NK T cells recognize GPIs. In addition, we note that the specificity of the anti-Vα14 mAb used by Schofield et al. (12) to show NK T cell expansion after GPI injection in vivo has been questioned by investigators in the field (7).

Our results differed from those of Schofield et al. (12), which showed significantly lower anti-CS Ab titers in CD1-deficient mice (mean titers of 1/8192 for wild-type vs 1/832 for CD1-deficient mice). Initially, we thought this difference could be related to the purity of the sporozoite preparation. Dissection of the salivary glands of infected *Anopheles* mosquitoes, which was the method employed by Schofield et al., can potentially result in the presence of mosquito-derived contaminants in the sporozoite preparation. Because GPI protein anchors are found from lower eukaryotes to mammals (28), we considered the possibility that mosquito-derived GPI anchors or other glycolipids might explain these contrasting results. Mosquito bites, on the other hand, are the natural mode of sporozoite delivery and generally ensure purer sporozoites for the immunization of mice than does their manual isolation from infected salivary glands before i.v. inoculation. Nevertheless, neither method of immunization revealed any significant differences between the anti-CS responses of wild-type and CD1-deficient mice, indicating that the contrasting results are not due to a mosquito-derived glycolipid and that the generation of IgG against the *P. berghei* CS protein is CD1d-independent. An alternative explanation that could also account for the different results obtained by Schofield et al. (12) relates to the heterogeneous genetic background of the CD1-deficient mice used (129/BALB/c mixture). Thus, it is still possible that the observed differences in the Ab response were governed by genetic disparities other than the CD1 mutation. However, experiments performed with MHC class II-deficient mice showing undetectable Ab response corroborate our claim that CD1d and NK T cells are not key elements for providing cognate help for anti-CS Ab responses. We observed no significant change of NKT phenotype and numbers in mice after immunization with irradiated sporozoites (data not shown). Furthermore, challenge experiments also support the notion that acquired anti-malarial immunity can develop in the absence of CD1d (our unpublished observations). These results also support a previous study (14) that showed immunization with a synthetic peptide representing the repeat region of the *P. berghei* CS protein failed to induce Abs in 11 strains of mice having different H-2 haplotypes, whereas immunization with a recombinant CS protein, containing the repeat plus short stretches of its flanking regions, overcame the unresponsiveness but with marked differences in Ab levels for the different strains tested.

In conclusion, despite the report that GPIs represent the main natural ligand presented by CD1d, our results demonstrate that they are not required for autorecognition of CD1d by Vα14$^+$ NK T cells or a broad panel of other CD1d-autoreactive T cells. Most importantly, our studies on IgG production upon sporozoite immunization do not support the hypothesis that CD1d and Vα14$^+$ NK T cells play a role in T cell help to the anti-CS response but rather indicate that B cell help is mediated through classical MHC class II/CD4$^+$ T cell interactions.

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