tion in promoting T cell proliferation in response to TCR and IL-2 activation. IL-2 receptor expression is not altered, and the T cells are able to produce cytokines in these STAT5A/B double knockout mice (*18*). It is possible that the TCR needs to directly activate STAT5 to induce some expression of immediate early genes, such as the CD69 gene, which has a role in T cell proliferation (*19*). Thus, our results suggest a route of TCR signaling in which STAT5 is directly activated and may contribute to early gene regulation and T cell proliferation.

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9. The Th2 clone D10 cells were maintained in Click's medium supplemented with fetal bovine serum (5%) and stimulated with B10BR splenocytes and hen egg conalbumin (0.1 mg/ml) (Sigma) every 3 to 4 weeks. For TCR cross linking, cells were kept on ice for 15 min, incubated with clonotypic antibody 3D3 to D10 TCR (5 μg/ml) on ice for an additional 15 min, washed, and treated with anti–mouse immunoglobulin G (Southern Biotechnology, Birmingham, AL; 8 μg/ml) at 37°C. Phospho-STAT5 analysis: Cells were collected by centrifugation and immediately lysed by boiling in electrophoresis loading buffer. Phosphorylation of STAT5 was detected by protein immunoblotting with specific antibody to phosphotyrosine 694 of STAT5 (New England Biolabs, Beverly, MA; Zymed, San Francisco, CA). IL-2 was obtained from Chiron (Emeryville, CA).
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11. 293T cells were transiently transfected with DNA by calcium phosphate precipitation. Experiments were carried out 2 days after transfection. Electrophoretic mobility-shift assay (EMSA) was done with whole-cell lysates that were incubated with nonspecific competitor DNA and ^{32}P-labeled β-casein SIE (5′-TGTGGACTTCTTGGAATTAAGGGACTTTTG-3′) or CD69-SIE (5′-GATCCGATTCCTGGAAAATG-3′). Protein-DNA complexes were resolved on 4% nondenaturing polyacrylamide gels in 0.5× tris-borate EDTA.
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13. Immunoprecipitation: 293T cell lysates were prepared in a lysis buffer containing Digitonin as detergent [4 × 10^6 cells, 250 μl of 1% Digitonin, 20 mM tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM NaF, aprotinin (10 mg/ml), leupeptin (10 mg/ml), pepstatin (1 mg/ml), and 250 μM o-Vanadate]. Lysates were incubated with anti-STAT5A (1.2 μg per sample; Santa Cruz Biotechnology, Santa Cruz, CA), prebound to protein A agarose beads, for 3 hours and washed two times in 1% Digitonin lysis buffer and two times in 0.1% Digitonin lysis buffer. T cell clone cells and primary T cells were lysed in a lysis buffer containing 1% Brij instead of Digitonin and supplemented with 10 mM $Na_4P_2O_7$ (10^7 cells, 250 μl). Anti-STAT5 and anti–CD3-ε (145-2C11) immunoprecipitations, agarose-conjugated anti-STAT5B, and anti–NF-κB (7.5 μg per sample) were added for 2 hours followed by washing steps. Protein immunoblots were done with anti-STAT5 (Santa Cruz Biotechnology), anti-phosphotyrosine (4G10), anti–TCR-α, anti–TCR-ζ, and anti-Lck (Santa Cruz Biotechnology).
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15. FLAG-tagged STAT5 was obtained by replacing the NH_2-terminal methionine of mouse STAT5A by FLAG-encoding sequence. In FLAG STAT5 Y694F, tyrosine (Y) 694 was changed to phenylalanine (F) (QuikChange Site-Directed Mutagenesis Kit; Stratagene). Expression of FLAG-tagged proteins was detected with antibody M2 (Kodak). Stable transfection of D10 cells was done as described (*12*). Linearized plasmid DNA (20 μg) was transfected by electroporation of antigen-stimulated D10 cells (2 × 10^7), followed by selection in a medium containing G418 (1.4 mg/ml) (Gibco-BRL). Stable clones were obtained about 1 1/2 months later, restimulated every 2 weeks, and expanded in the presence of IL-2 (3 U/ml).
16. Proliferation assay: D10 cells and transfectants (2.5 × 10^4) were incubated with mitomycin C–treated splenocytes of B10.BR mice (5 × 10^4) and the agonistic peptide CA134-146. After 60 hours, 1 μCi of [^{3}H]thymidine was added for 4 hours, followed by harvest and liquid scintillation counting.
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CD1d-Restricted Immunoglobulin G Formation to GPI-Anchored Antigens Mediated by NKT Cells

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Immunoglobulin G (IgG) responses require major histocompatibility complex (MHC)–restricted recognition of peptide fragments by conventional CD4$^+$ helper T cells. Immunoglobulin G responses to glycosylphosphatidylinositol (GPI)-anchored protein antigens, however, were found to be regulated in part through CD1d-restricted recognition of the GPI moiety by thymus-dependent, interleukin-4–producing CD4$^+$, natural killer cell antigen 1.1 [(NK1.1)$^+$] helper T cells. The CD1-NKT cell pathway regulated immunogobulin G responses to the GPI-anchored surface antigens of *Plasmodium* and *Trypanosoma* and may be a general mechanism for rapid, MHC-unrestricted antibody responses to diverse pathogens.

NKT cells are unusual CD4$^+$, NK1.1$^+$ lymphocytes (*1*) that produce interleukin-4 (IL-4) rapidly in response to T cell receptor (TCR) ligation (*2*). These cells have a skewed $V_\alpha V_\beta$ TCR repertoire (V_α14 and V_β8 in mice) (*3*), suggesting that they are positively selected by a limited range of ligands. Murine NKT cells are positively selected by cortical thymocytes expressing the non-MHC–encoded but MHC class I–like molecule CD1d (*1*). The related human CD1b and CD1c molecules can elicit cytolytic and interferon-γ responses by presenting mycobacterial glycolipid antigens to CD8$^+$ or CD4$^-$CD8$^-$ T cells (*4*). Murine V_α14$^+$ NKT cells recognize synthetic α-galactosylceramide in the context of CD1d (*5*), and murine CD1d in transfected human T2 cells associates with phosphatidylinositol (PI)-containing compounds that may be GPIs (*6*). Therefore, CD4$^+$ NKT cells

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may participate in CD1d-restricted recognition of lipid antigens. However, the natural ligand and functional significance of NKT cells in immune responses in vivo remains unclear. The present study was initiated to test the assumption of MHC restriction of immunoglobulin G (IgG) responses to the GPI-anchored surface antigens of important protozoal pathogens. We have found instead that several such responses are controlled in part by CD1d-restricted recognition of GPI moieties by $CD4^+$ NKT cells.

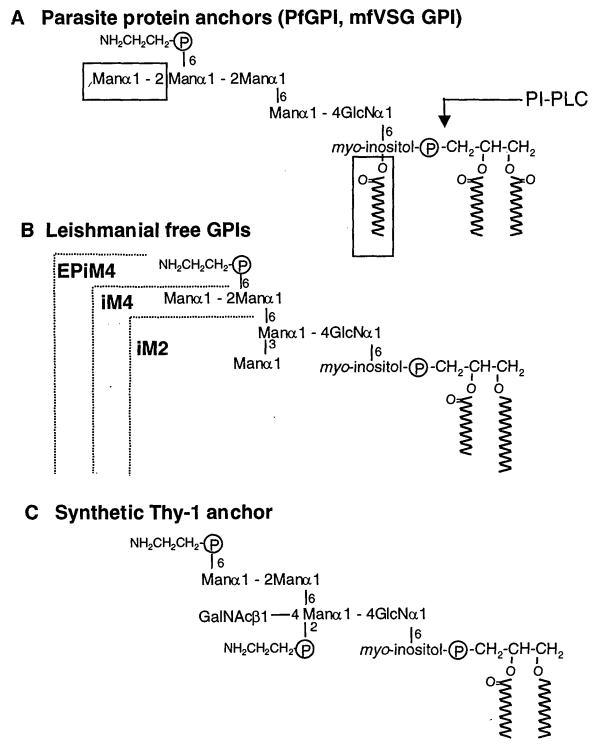

Fig. 1. Diagrammatical representation of GPI structures used in this study. Purification and compositional analyses are as described (*12–14*). (**A**) COOH-terminal GPIs from *T. brucei* and *P. falciparum*. Boxed areas represent modifications found in PfGPI. The cleavage site of mfVSG by phosphatidylinositol-specific phospholipase C (PI-PLC) is indicated. (**B**) Free iM2, iM4, and EP-iM4 GPIs of *L. mexicana*. Nomenclature is as described (*14*), where all isomers contain one mannose in α1-3 linkage, EP indicates ethanolamine phosphate, and M2 and M4 indicate the number of mannose residues, as shown. (**C**) Chemically synthesized rat brain Thy-1 GPI.

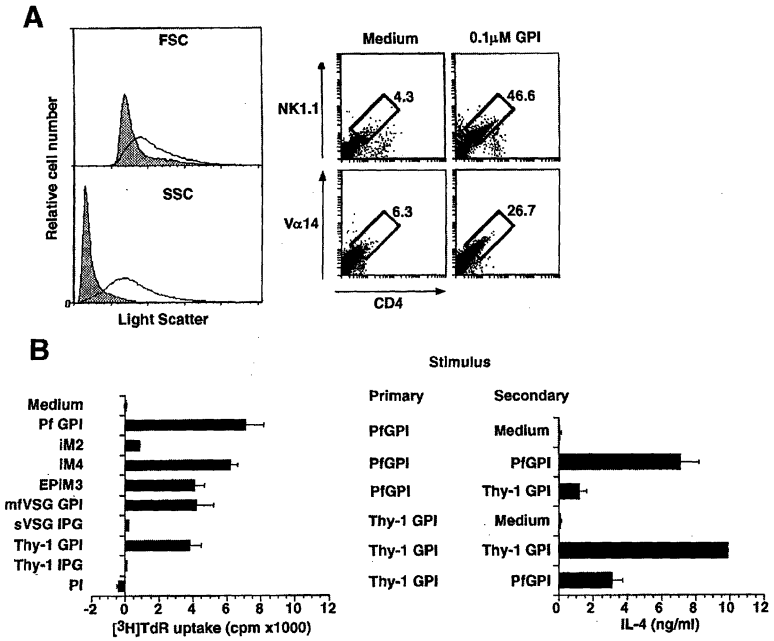

Fig. 2. Response of peripheral NKT cells to purified GPIs in vitro. (**A**) As determined by forward (FSC) and side (SSC) light scatter, splenocytes from SPZ-primed class $II^{-/-}$ donors proliferate within 48 hours exposure to PfGPI (unshaded) compared with medium controls (shaded). The responding cells are $NK1.1^+$, $CD4^+$ and include a $V_\alpha14^+$, $CD4^+$ subset (*25*). (**B**) Splenocytes from class $II^{-/-}$ donors were exposed to various antigens, and [^{3}H]TdR incorporation was determined after 4 days. Other cultures were exposed to PfGPI or Thy-1 GPI for 4 days, washed, and cultured in IL-2 (10 U/ml) for 2 days, followed by replating with irradiated wild-type APCs and restimulation with either PfGPI or Thy-1 GPI for 48 hours. IL-4 levels in the supernatant were determined by capture ELISA.

T cell–dependent IgG responses to protein antigens are thought to be exclusively MHC class II–restricted. However, allogeneic bone-marrow irradiation chimeras were similar to syngeneic controls in responding to malaria sporozoites (SPZs) with IgG to the circumsporozoite (CS) protein, despite being unable to respond to the nominal protein antigens tetanus toxoid (TT) or a full-length recombinant *Plasmodium falciparum* CS protein (recCS) (*7*). Nude mice cannot respond to SPZs with anti-CS IgG, and passive transfer of depleting antibodies to CD4 into euthymic animals abolishes the anti-CS response to SPZs (*8*). However, nude mice engrafted with irradiated neonatal allogeneic thymi mounted anti-CS IgG responses to SPZs similar to those of recipients of syngeneic thymi, but did not respond to recCS or TT (*7*). Mice lacking both class II and class II–restricted $CD4^+$ T cells, and that are unable to respond to T-dependent antigens (*9*), produced anti-CS IgG (mean $\log_2$ reciprocal titer of 10) in response to SPZs (*10*). Thus, $CD4^+$ T cells are required for the IgG response to the native CS protein, but this may proceed through a MHC class II–independent route.

The native CS protein is posttranslationally modified by a GPI anchor (*11*), whereas TT and recCS are not. To determine whether the GPI anchor accounts for the difference in immunological behavior of the proteins, we purified COOH-terminal GPIs from affinity-purified GPI-anchored proteins of *P. falciparum* (PfGPI) and *Trypanosoma brucei* membrane-form variant surface glycoprotein (mfVSG) (*12*) (Fig. 1A), and nonprotein-linked free GPIs from *Leishmania mexicana* (*12*, *13*) (Fig. 1B). The compositional purity of these latter molecules was confirmed by gas chromatography–mass spectrometry (GC-MS). In addition, a phosphorylated and lipidated mammalian GPI based on the rat brain Thy-1 GPI (Fig. 1C), and the corresponding inositolphosphoglycan (IPG) lacking a lipid tail, both chemically synthesized by *n*-pentenyl glucoside strategy and compositionally pure by ^{1}H nuclear magnetic resonance (NMR) analysis (*14*), were also used. To generate responses to the hapten fluorescein (FLU), native and synthetic GPIs and IPG were exposed to 2-iminothiolane to introduce a sulfhydryl onto free amino groups, desalted, and conjugated in a molar ratio of 1:1 to fluoresceinated, maleimide-activated ovalbumin (OVA^{FLU}). In contrast to sham-OVA^{FLU} alone or sham-OVA^{FLU} mixed with equal molar amounts of free PfGPI, PfGPI-OVA^{FLU} conjugates were able to induce anti-FLU IgG1 formation in MHC class $II^{-/-}$ mice (mean $\log_2$ reciprocal titer of 8). Similar IgG responses

were obtained in class $II^{-/-}$ mice with the mfVSG of *T. brucei*, but not the deacylated soluble VSG derived by PI-specific phospholipase C (PI-PLC) hydrolysis (Fig. 1A), demonstrating that the GPI lipid domain is required, and the GPI glycan is not sufficient, for the phenomenon. This was confirmed by comparing responses to OVA^{FLU} conjugated to either synthetic Thy-1 GPI or Thy-1 IPG lacking fatty acid (log_2 reciprocal titer of 9.75 versus no response, respectively). Thus, IgG responses in class $II^{-/-}$ mice require linkage of antigen to GPI with an intact lipid, which may be composed of diacylglycerol or alkylacylglycerol.

Although lacking conventional T cells, class $II^{-/-}$ mice retain a diverse population of MHC-nonrestricted $CD4^+$ α/β TCR^+ T cells, including NKT cells and other $CD4^+$ cells selected on CD1 (*9*, *15*). We therefore hypothesized that IgG responses to native and synthetically conjugated GPI-anchored proteins in both wild-type and class $II^{-/-}$ mice proceed from CD1d-restricted presentation of GPIs to nonconventional T cells. To test this hypothesis, we examined the in vitro proliferative and cytokine responses to purified malarial GPI of splenocytes from animals primed with malaria SPZs. There was a marked increase in both the relative and absolute numbers of $NK1.1^+$ $CD4^+$ blastoid cells responding to GPI from both class $II^{-/-}$ (Fig. 2A) and wild-type mice. A high frequency of both $V_\alpha14^+$ $CD4^+$ (Fig. 2A) and $V_\beta8^+$ cells was also detected in the responding population. No exogenous cytokines were required for this proliferation, but supplementation of cultures with IL-2 (5 U/ml) increased the level of response.

To examine the fine specificity of responding cells, we exposed splenocytes from wild-type and class $II^{-/-}$ animals primed to *P. berghei* SPZs to 0.5 μM of the GPI structures shown in Fig. 1, together with dipalmitoyl-PI. The cells responded to a similar degree to most intact GPIs, but only weakly to the iM2 GPI with truncated glycan and not at all to glycans lacking the fatty acid domain, or to PI (Fig. 2B). Thus, both glycan and fatty acids are required for recognition, and NKT cells from SPZ-primed donors respond to a range of GPIs from diverse protozoal and mammalian taxa. However, because the full range of structures presented to the host under these priming conditions is not known, the results may reflect either broad recognition of diverse antigens by the general population of NKT cells or heterogeneous responses from a clonally mixed population. To further distinguish between these possibilities, cells were expanded in the presence of either PfGPI or Thy-1 GPI for 4 days, rested in IL-2, then restimulated with homologous or heterologous antigen. Cells expanded by either antigen responded significantly less well to the heterologous stimulus (Fig. 2B). Analysis of antigen-specific frequencies after in vitro culture revealed no increase above background levels in response to PfGPI in naïve donors, but a clear increase in antigen-reactive NKT cells from SPZ-primed donors (7 to 30% of total) (*16*). However, naïve and primed donors both mounted significant responses to the iM4 GPI (to 5% of NKT cells) (*16*). Together, the data are consistent with the reported fine specificity of CD1-restricted $CD4^-CD8^-$ human T cells for the glycan component of glycolipids (*4*, *17*), suggesting that antigen priming expands clonally diverse NKT cells that are able to discriminate among structurally distinct GPIs, but that high precursor frequencies for some GPIs may occur even among naïve animals kept under specific-pathogen–free conditions.

Cells that have been positively selected on NK1.1 and CD4 proliferate and produce cytokines specifically in response to TCR-mediated signals (*2*, *18*). When sorted $NK1.1^+$ $CD4^+$ cells from wild-type and class $II^{-/-}$ mice that had been primed to SPZs were cultured with irradiated wild-type or class $II^{-/-}$ antigen-presenting cells (APCs), they responded to purified GPIs, as determined by incorporation of [^{3}H]thymidine ([^{3}H]TdR) and the production of high levels of IL-4 (Fig. 3A). No proliferation in the absence of APCs indicated that GPIs do not provide a direct activation signal to NKT cells that is sufficient to induce cell growth. NKT cells did not respond to GPIs when cultured with irradiated APCs from β_2-microglobulin$^{-/-}$ ($\beta_2M^{-/-}$) and CD1.1/CD1.2$^{-/-}$ ($CD1^{-/-}$) (*19*) donors, or with wild-type and class $II^{-/-}$ APCs in the presence of anti-CD1.1 (1B1) (*20*), but responded fully in the presence of isotype controls (Fig. 3A). The proliferative and IL-4 response to PfGPI of NKT cells and the $V_\alpha14^+$, $CD4^+$ subset in unfractionated splenocytes could also be blocked by the anti-CD1 monoclonal antibody 1B1 (Fig. 3B). Thus, the recognition of GPIs by NKT cells is MHC-independent and CD1-restricted. In addition, NKT cells produced IL-4 in response to CD1.1-transfected J774 macrophages (*20*) in the absence of exogenous antigen, but not in response to sham-transfected controls. Nonetheless, the response was enhanced when CD1.1-transfectants were pulsed with PfGPI (Fig. 3A). The response of NKT cells to CD1.1, observed previously (*5*, *18*), has been adduced in support of the proposition that this cell population may play a physiological role in the absence of associative recognition of antigen (*5*, *21*). However, as reported (*5*, *18*), in the absence of exogenous antigen no cytokine expression was detected in response to APCs expressing normal levels of CD1.1. Thus, high levels of CD1.1 expression in transfected cells may alone be sufficient to drive proliferation. Alternatively, because NKT cells can respond to mammalian GPIs [for example, syn-

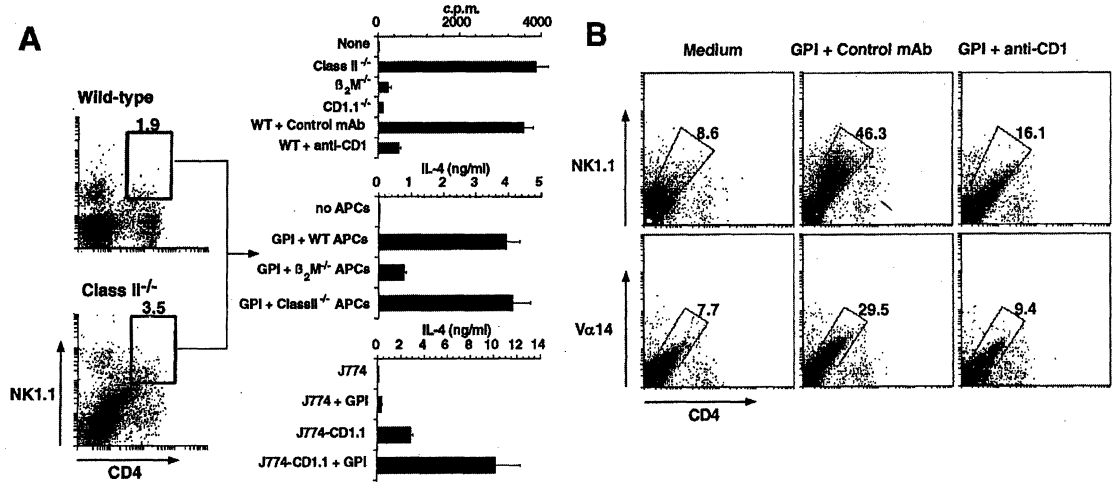

Fig. 3. The proliferative and IL-4 response of NKT cells to PfGPI is MHC-independent and CD1-restricted. (**A**) Sorted $NK1.1^+$, $CD4^+$ cells (2×10^4) from wild-type or class $II^{-/-}$ donors were placed in triplicate with or without purified GPI on irradiated splenocyte APCs from wild-type (WT), class $II^{-/-}$, $\beta_2M^{-/-}$, or $CD1^{-/-}$ donors, or CD1.1-transfected and sham-transfected J774 macrophages. [^{3}H]TdR incorporation was determined after 3 days, or IL-4 production in the presence or absence of anti-CD1 was determined as in Fig. 2. (**B**) Splenocytes from SPZ-primed class $II^{-/-}$ donors were exposed to PfGPI in the presence or absence of anti-CD1 or isotype control and taken for flow cytometric analysis after 3 days.

thetic Thy-1 GPI (Fig. 2B)], CD1.1-transfectants may be able to present endogenous GPIs to NKT cells, as suggested by the association of CD1d in transfected T2 cells with PI-containing compounds (*5*). Thus, CD1 may not be "empty," and self-reactive NKT cells may arise through incomplete negative selection. Such a possibility may explain the nonantigen-specific regulatory activity of NKT cells (*2*, *22*).

NKT cells can induce Ig class switch in B cells exposed to anti-IgD (*2*). Extending to antigen-specific systems, NKT cells cooperated with B cells by ELISPOT assay in CD1-restricted IgG formation to GPI-OVA^{FLU} and native *P. berghei* CS protein formation, but not to OVA^{FLU} (Fig. 4A). To determine, therefore, whether CD1.1- or CD1.2-restricted antibody formation was a major or minor contributor to the IgG response to GPI-anchored proteins and SPZs in vivo, we exposed CD1$^{-/-}$ mice and wild-type controls to mfVSG^{FLU}, SPZs, or recCS. Responses to mfVSG^{FLU} and SPZs were significantly curtailed in CD1$^{-/-}$ mice (Fig. 4B), indicating that under these conditions the CD1-restricted pathway of IgG formation is an important component of responses to the native CS protein. Both groups responded equally to recCS, confirming that class II–dependent responses are unaffected by loss of CD1. It is not yet clear whether the IgG responses to SPZs in CD1$^{-/-}$ mice result from class II–restricted responses to the intact GPI-anchored CS protein or to a proportion of the antigen adventitiously deacylated in these preparations, or from a non–class II, non-CD1 pathway.

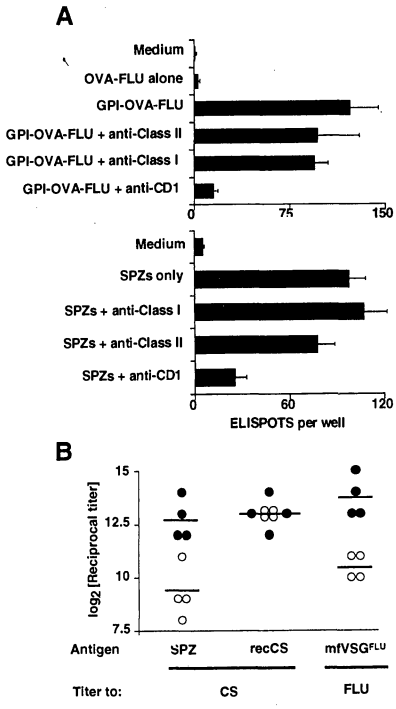

Fig. 4. CD1-restricted antibody formation to neo-GPI-proteins and malaria SPZs. (**A**) Donor *nu/nu* mice were primed twice with *P. berghei* SPZs or twice with LPS^{FLU}. Splenocytes were cultured in the presence of IL-2 (10 U/ml), with and without antigen (0.1 μg/ml sham-OVA^{FLU}, PfGPI-OVA^{FLU}, or 5 × 10^4 SPZs), anti-class I, anti-class II, and anti-CD1, with 10^4 NKT cells from SPZ-primed class II$^{-/-}$ donors. Antigen-specific IgG production was quantified by ELISPOT against fluoresceinated dog serum albumin for responses to OVA^{FLU}, and rCS for responses to SPZs. (**B**) Responses of CD1.1/CD1.2$^{-/-}$ (○) and Balb/c wild-type mice (●) to SPZs, recCS, and mfVSG^{FLU}. IgG titers were determined as described (*7*).

GPIs are widespread among eukaryotes, and the expression of GPI-anchored proteins and free GPIs is particularly abundant among the parasitic protozoa. Because CD1-restricted NKT cells can recognize GPIs from diverse taxa (Fig. 2B), CD1-restricted IgG formation may represent a general mechanism for rapid responses to the GPI-anchored surface antigens of various pathogens. The native CS protein appears to be more immunogenic than the recombinant version. Inoculation of as few as 10^4 nonreplicating irradiated SPZs (1 ng of native CS protein, assuming 10^6 copies per cell) is sufficient to elicit an antibody response comparable to that obtained with 1 to 10 μg of recCS. The rapid responses of NKT cells in vivo (*2*) and a relatively high precursor frequency of antigen-specific NKT cells may contribute to this phenomenon. Thus, consistent with the "danger model" of pathogen-initiated immune responses (*23*), CD1-restricted immunity may be intermediate between the innate "pattern recognition" and adaptive immune systems. MHC-restricted nonresponsiveness to malarial surface antigens has been proposed to be a major obstacle to the development of vaccines (*24*). Because both human and murine CD1 molecules are relatively nonpolymorphic, GPI anchors may provide universal T cell sites, overcoming MHC restriction in antibody responses to various pathogens.

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7. Irradiated H-2 congenic mice received 10^7 T cell–depleted syngeneic or allogeneic bone-marrow cells. Thymic lobes from neonatal Balb/c, Balb/B, and Balb/K mice were irradiated and implanted into adult Balb/c *nu/nu* mice. Mice were rested 12 weeks before testing for chimerism. *P. falciparum* and *P. berghei* SPZs were dissected from *Anopheles freeborni* and *A. stephensi*, respectively. Some preparations were kept at −70°C before use. Mice were primed with TT, recCS, or SPZs; boosted after 1 week, and IgG responses analyzed by enzyme-linked immunosorbent assay (ELISA). RecCS, derived from the T4 strain and consisting of the entire sequence except for the COOH-terminal 21 amino acids (GPI-anchor signal sequence), was used as immunizing antigen and to detect responses to *P. falciparum* SPZs. The *P. berghei* rCS (detection antigen only for *P. berghei* SPZs) encompasses amino acids 81 to 277 (*26*). Antigen-specific end titers were defined as the last titration giving values statistically different from binding to plates without antigen. A $\log_2$ reciprocal titer of 4.0 was the background cutoff. To TT, recCS, and *P. berghei* SPZs, syngeneic thymic chimeras mounted mean $\log_2$ reciprocal IgG titers of 15.14, 13.5, and 13.5, respectively. The equivalent values for allogeneic chimeras were 4.5, 4.25, and 13. For syngeneic bone-marrow chimeras the equivalent values were 15.11, 11.25, and 14.6, and for allogeneic chimeras 8.5, 7.25, and 15.25, respectively.
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Grassland Vegetation Changes and Nocturnal Global Warming

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Global minimum temperatures (T_{MIN}) are increasing faster than maximum temperatures, but the ecological consequences of this are largely unexplored. Long-term data sets from the shortgrass steppe were used to identify correlations between T_{MIN} and several vegetation variables. This ecosystem is potentially sensitive to increases in T_{MIN}. Most notably, increased spring T_{MIN} was correlated with decreased net primary production by the dominant C_4 grass (*Bouteloua gracilis*) and with increased abundance and production by exotic and native C_3 forbs. Reductions in *B. gracilis* may make this system more vulnerable to invasion by exotic species and less tolerant of drought and grazing.

There is general consensus that there is an anthropogenic warming signal in the long-term climate record (*1*). Over land, this is primarily due to average annual minimum temperatures (T_{MIN}) having increased at twice the rate of maximum temperatures (T_{MAX}) (*1*, *2*). At the global scale, these increases in T_{MIN} are related to increases in global cloudiness (*1*, *3*). Experiments with agricultural plants and insect pests suggest important roles for T_{MIN} in influencing plant and insect development (*4*, *5*). However, there has been little research on the consequences of elevated T_{MIN} for natural ecosystems (*6*, *7*). If elevated T_{MIN} leads to longer growing seasons, net primary production and carbon sequestration may increase as a consequence (*8*). However, the opposite may occur if elevated T_{MIN} leads to increased plant and microbial nocturnal respiration rates without a compensatory increase in photosynthesis. Additionally, elevated T_{MIN} could shift competitive interactions among C_3 (cool-season) and C_4 (warm-season) plants.

It is important to identify features of ecosystems that are sensitive to changes in T_{MIN}. To date, most modeling efforts and experimental manipulations investigating ecosystem responses to climate change have assumed that future warming will occur primarily during the day or uniformly over the diurnal cycle. This assumption clearly is not valid on a global level nor at most regional scales (*2*). Furthermore, there is no a priori reason to assume that ecosystems will respond similarly to changes in T_{MIN} and T_{MAX}. To investigate potential ecological consequences of elevated T_{MIN}, we examined a 23-year data set for correlations between temperature [T_{MIN}, T_{MAX}, and mean annual temperature (T_{AVE}) (T_{AVE} = (T_{MIN} + T_{MAX})/2)] and both the abundance and aboveground net primary productivity (ANPP) of several key plant species and functional groups found at the Central Plains Experimental Range (*9*) in northeastern Colorado.

We identified seasonal and annual trends in T_{MIN} and T_{MAX} to determine whether asymmetric diurnal temperature increases held true for this site (*10*). The densities of most species were determined by counting all individuals within permanently marked quadrats (*11*). Harvests at time of peak standing crop were used as estimates of ANPP (*12*, *13*). Plants in the shortgrass steppe are commonly water-limited, and variation in precipitation could obscure plant responses to gradually changing temperatures (*9*, *14*). Therefore, we included annual and seasonal precipitation totals, in addition to annual and seasonal minimum and maximum temperatures, as variables for stepwise regression model selection (*15*). We constructed linear models to evaluate significant correlations between these variables and ANPP or plant species density (*16*).

Mean annual temperatures (T_{AVE}) have increased by an average of 0.12°C year^{-1} at this site since 1964 (P = 0.0001, R^2 = 0.52). During this period, T_{MAX} increased 0.085°C year^{-1} (Fig. 1A), whereas T_{MIN} increased 0.15°C year^{-1} (Fig. 1B). We limited further analyses of temperature to the period beginning in 1970, when standardized monitoring of vegetation density was initiated. Since 1970, T_{AVE} has risen over 1.3°C, largely due to a significant increase in T_{MIN} of 0.12°C year^{-1} (P = 0.003; R^2 = 0.44). However, there was no significant trend for T_{MAX} (P = 0.49). Averages of seasonal minimum temperatures since 1970 also exhibited significant warming, with similar trends in winter (0.17°C year^{-1}, P = 0.0013, R^2 = 0.40), spring (0.16°C year^{-1}, P = 0.0007, R^2 = 0.43), and summer T_{MIN} (0.12°C year^{-1}, P = 0.004, R^2 = 0.33). No significant trends were detected in fall T_{MIN} (P = 0.64, R^2 = 0.01). Annual precipitation (Fig. 1C) varied from 230 to 480 mm and has also exhibited a significant linear increase since 1970 (6 mm year^{-1}, P = 0.007, R^2 = 0.30). However, there were no significant correlations between annual or seasonal T_{MIN} and annual or seasonal precipitation (P > 0.1).

Since 1983 (*12*), ANPP of *Bouteloua gracilis*, the dominant C_4 grass of the shortgrass steppe, declined over time (–12.2 g m^{-2} year^{-1}; P = 0.002; R^2 = 0.78), and was negatively correlated with average spring T_{MIN} (Fig. 2A). ANPP of the most abundant C_3 forb, *Sphaeralcea coccinea*, was negatively correlated with winter T_{MIN} (Fig. 2B). In contrast, ANPP of

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