STAT5 Interaction with the T Cell Receptor Complex and Stimulation of T Cell Proliferation

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The role of STAT (signal transducer and activator of transcription) proteins in T cell receptor (TCR) signaling was analyzed. STAT5 became immediately and transiently phosphorylated on tyrosine 694 in response to TCR stimulation. Expression of the protein tyrosine kinase Lck, a key signaling protein in the TCR complex, activated DNA binding of transfected STAT5A and STAT5B to specific STAT inducible elements. The role of Lck in STAT5 activation was confirmed in a Lck-deficient T cell line in which the activation of STAT5 by TCR stimulation was abolished. Expression of Lck induced specific interaction of STAT5 with the subunits of the TCR, indicating that STAT5 may be directly involved in TCR signaling. Stimulation of T cell clones and primary T cell lines also induced the association of STAT5 with the TCR complex. Inhibition of STAT5 function by expression of a dominant negative mutant STAT5 reduced antigen-stimulated proliferation of T cells. Thus, TCR stimulation appears to directly activate STAT5, which may participate in the regulation of gene transcription and T cell proliferation during immunological responses.

Specific antigen stimulation of the TCR induces tyrosine phosphorylation of signaling proteins, which results in proliferation and differentiation of T lymphocytes (*1*). Lck, a Src family tyrosine kinase, mediates tyrosine phosphorylation of conserved motifs called ITAMs (immune receptor tyrosine-based activation motifs) (*1–3*) in the invariant chains of the TCR. Through interaction with the phosphorylated ITAM sites, many signaling proteins are recruited by the TCR complex, resulting in activation of various pathways, including activation of protein kinase ZAP-70, phospholipase C–γ, mitogen-activated protein kinase, and calcium mobilization (*1–3*). Furthermore, TCR signaling results in immediate activation of transcription factors, including NF-AT and Rel family proteins, which participate in regulation of cytokine genes (*4*).

STAT proteins are transcription factors that induce immediate early gene expression (*5*). During cytokine signaling, STATs bind to phosphotyrosine containing motifs of activated cytokine receptors through their SH2 domains and are phosphorylated by members of the JAK family (*5*). However, other protein tyrosine kinases (PTKs), including the epidermal growth factor receptor and Src, can also phosphorylate and activate STAT proteins (*6*). STAT5A knockout mice show some defects in T cell proliferation and function (*7*). However, it is unclear whether STAT proteins directly take part in TCR signaling or are activated directly by PTKs during TCR activation.

To study TCR-induced STAT phosphorylation, we used the T helper cell clone D10, in which TCR signaling is strongly induced by the clonotypic TCR antibody 3D3 (*8*). These cells do not generate interleukin-2 (IL-2) in response to TCR activation (*8*); therefore, the role of TCR signaling can be examined without endogenously produced IL-2. Cells were treated with antibody to the TCR (anti-TCR), IL-2, or interferon-γ (IFN-γ). Extracts from treated or control cells were analyzed with an antibody specific for tyrosine-phosphorylated STAT5 (Fig. 1) (*9*). Treatment of cells with anti-TCR resulted in an increase in the tyrosine phosphorylation of STAT5 within 7 min. More extensive tyrosine phosphorylation of STAT5 was induced by treatment of cells with IL-2 for 15 min. IFN-γ activated STAT1 in D10 cells (*10*) but did not induce STAT5 phosphorylation. The blot was reprobed with anti-STAT5 to verify that about equal amounts of STAT5 were present in the samples analyzed.

To examine whether Lck could mediate the activation of STAT5, we transfected 293T cells with expression vectors for Lck, STAT5A, STAT5B, and STAT1 and analyzed them for STAT DNA-binding activities induced by Lck (*11*). Cells that were transfected with STATs alone did not show any activity (Fig. 1B). However, a DNA-binding complex was induced when cells were cotransfected with either STAT5A or STAT5B with Lck. The presence of STAT5A or STAT5B in the complex was confirmed by observation of supershifted complexes generated by incubation with specific antibodies to STAT5A or STAT5B. Lck did not induce binding of STAT1 to the beta-casein promoter–derived DNA probe.

To confirm the functional role of Lck in STAT5 activation, we examined D10 cells and Lck-deficient D10 cells that were generated by overexpression of antisense transcripts of Lck (*12*). STAT5 complexes were induced with anti-TCR in the parental D10 cells in 7 min (Fig. 1C). Complex formation was diminished in Lck-deficient D10 cells under the same conditions, suggesting a necessary role for Lck in STAT5 activation. The lack of Lck protein expression in Lck-deficient cells was confirmed by protein immunoblot analysis. STAT5 activation by IL-2 was substantially stronger than that by TCR stimulation in the same cells. In contrast, a complex representing constitutive activity of STAT6 in these cells appeared not to be affected in the absence of Lck. These data indicated that STAT5 was specifically activated in a Lck-dependent manner immediately after TCR stimulation.

A motif YxxL(I/V) (where Y is Tyr, L is Leu, I is Ile, and V is Val), which is a STAT5-docking site in a variety of cytokine receptors, resembles the conserved YxxL motif in ITAM. This finding indicated a possibility of direct interaction of STAT5 with the tyrosine-phosphorylated signal-transducing chains of the TCR. To address this question, we expressed Lck, TCR-ζ, and STAT5 in 293T cells by transfection of respective expression vectors. First, we analyzed tyrosine phosphorylation of each protein in the lysates from cells transfected with these vectors individually or in combination (Fig. 2A). TCR-ζ was not detectably phosphorylated in the absence of Lck but became phosphorylated on tyrosine in the presence of Lck. Transfection with Lck alone showed that there is no protein endogenously present and migrating at the position of TCR-ζ. The STAT5 protein was also tyrosine phosphorylated when cotransfected with Lck and the ζ chain. Phosphorylated TCR-ζ chain was coimmunoprecipitated with STAT5 only when Lck was coexpressed (Fig. 2B) (*13*). The identity of tyrosine-phosphorylated ζ chain, which was coimmunoprecipitated with an anti-STAT5, was confirmed by reblotting with an antibody to the ζ

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chain. In the absence of Lck, STAT5 and TCR-ζ were not tyrosine phosphorylated or associated with one another. The same membrane was reblotted again with anti-STAT5 showing equal amounts of STAT5 in coimmunoprecipitation (bottom panel). In contrast, Lck itself was not detected in the STAT5–TCR-ζ chain complex (Fig. 2B), suggesting that Lck only transiently phosphorylated the ζ chain, which enabled the ζ chain to serve as a docking protein for STAT5. When the experiment was done in the absence of the STAT5A cDNA expression plasmid, precipitation of phosphorylated TCR-ζ was not detected under this condition. Three repetitions of the experiment gave similar results. The other antibodies to the COOH-terminus or the NH_2-terminus of STAT5 gave the same results (*10*).

Furthermore, although Lck action was to generate tyrosine-phosphorylated docking sites within the associated TCR chains, which serve to bring STAT5 into the vicinity of TCR, the existence of STAT5-docking sites in the TCR complex may also in turn facilitate the phosphorylation of STAT5 by Lck itself or by other PTKs. To examine this possibility, we used limiting amounts of Lck and analyzed whether its function in STAT5 phosphorylation could be augmented by the presence of the ζ chain. In this experiment, cells were transfected with varied DNA concentrations of Lck and a constant concentration of STAT5 and the ζ chain to determine the minimal amount of Lck that was required for phosphorylation of STAT5 either in the absence or in the presence of the ζ chain (Fig. 2C). STAT5 was then immunoprecipitated and analyzed for tyrosine phosphorylation by protein immunoblotting. In the absence of the ζ chain, STAT5 phosphorylation was detected only with the largest amount of Lck cDNA transfected (Fig. 2C). In contrast, in the presence of the ζ chain, maximal STAT5 phosphorylation was observed even at the lowest concentration of Lck cDNA. Thus, TCR-ζ contributes to enhancement of tyrosine phosphorylation of STAT5 in this system. In the absence of Lck, no such enhancement was observed.

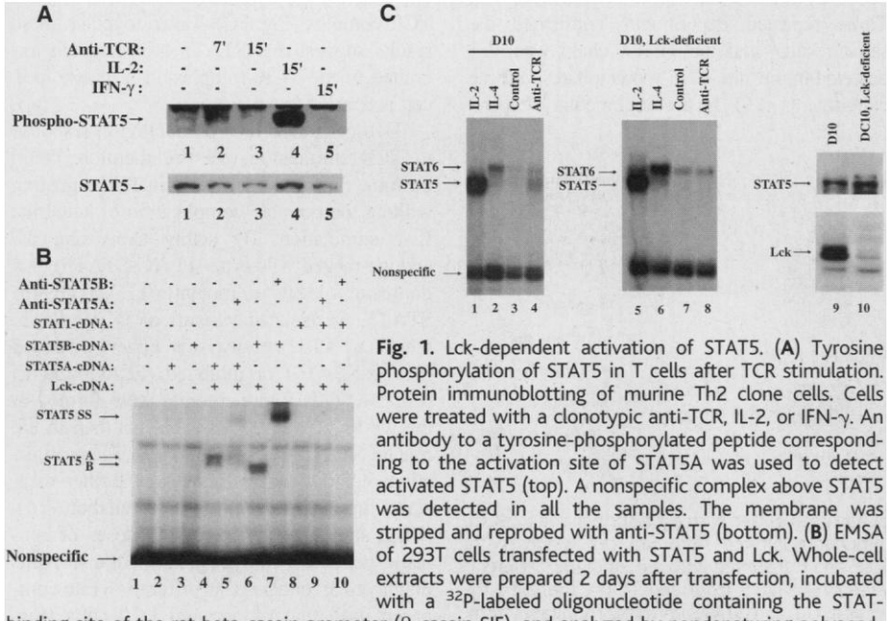

Fig. 1. Lck-dependent activation of STAT5. (**A**) Tyrosine phosphorylation of STAT5 in T cells after TCR stimulation. Protein immunoblotting of murine Th2 clone cells. Cells were treated with a clonotypic anti-TCR, IL-2, or IFN-γ. An antibody to a tyrosine-phosphorylated peptide corresponding to the activation site of STAT5A was used to detect activated STAT5 (top). A nonspecific complex above STAT5 was detected in all the samples. The membrane was stripped and reprobed with anti-STAT5 (bottom). (**B**) EMSA of 293T cells transfected with STAT5 and Lck. Whole-cell extracts were prepared 2 days after transfection, incubated with a ^{32}P-labeled oligonucleotide containing the STAT-binding site of the rat beta-casein promoter (β-casein SIE), and analyzed by nondenaturing polyacrylamide gel electrophoresis (PAGE). STAT5A- or STAT5B-specific antibodies were added to the protein-DNA–binding reactions where indicated (lanes 5, 7, 9, and 10). Cells were transfected with cDNAs for Lck and either STAT5A, STAT5B, or STAT1. The positions of protein-DNA complexes are marked. STAT5-SS: Antibody-induced supershift complex of STAT5. (**C**) Critical role for Lck in STAT5 activation by TCR stimulation. Lysates prepared from parental D10 and Lck-deficient D10 cells were subjected to EMSA with β-casein SIE as the probe. Cells were treated with clonotypic anti-TCR. As a control, cells were also treated with IL-2 or IL-4 for 17 min (lanes 1, 2, 5, and 6). The protein blot of D10 or Lck-deficient D10 cells was probed with anti-STAT5 (top) or anti-Lck (bottom, lanes 9 and 10).

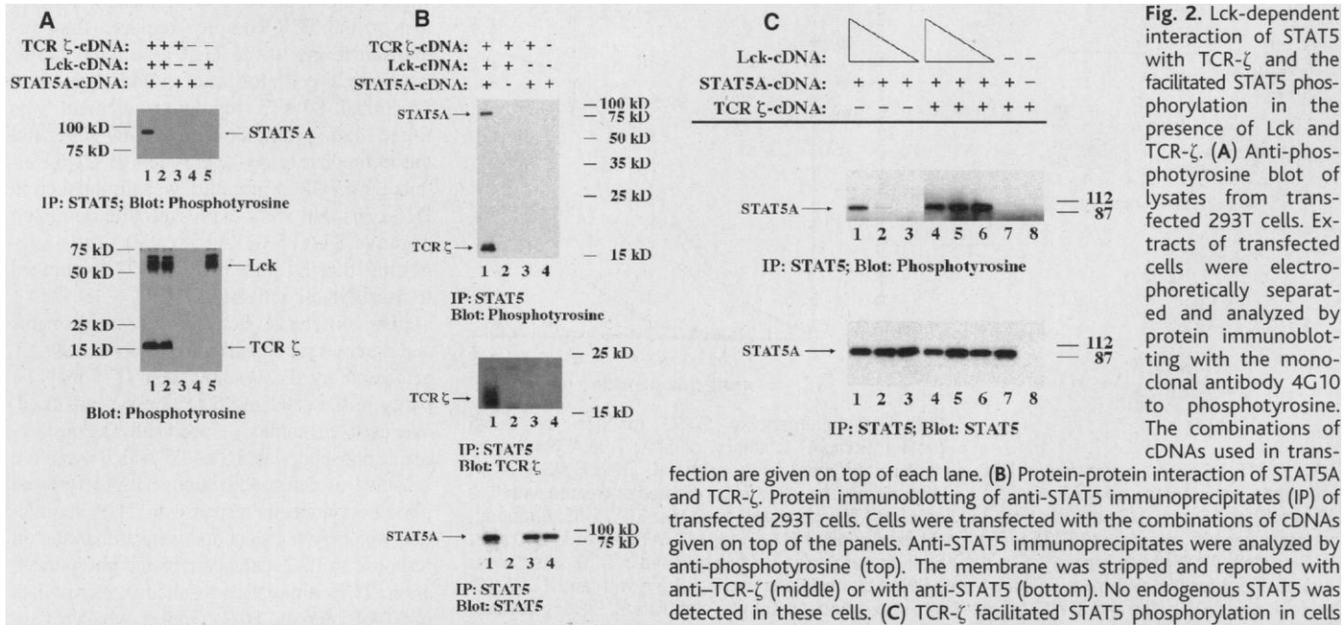

Fig. 2. Lck-dependent interaction of STAT5 with TCR-ζ and the facilitated STAT5 phosphorylation in the presence of Lck and TCR-ζ. (**A**) Anti-phosphotyrosine blot of lysates from transfected 293T cells. Extracts of transfected cells were electrophoretically separated and analyzed by protein immunoblotting with the monoclonal antibody 4G10 to phosphotyrosine. The combinations of cDNAs used in transfection are given on top of each lane. (**B**) Protein-protein interaction of STAT5A and TCR-ζ. Protein immunoblotting of anti-STAT5 immunoprecipitates (IP) of transfected 293T cells. Cells were transfected with the combinations of cDNAs given on top of the panels. Anti-STAT5 immunoprecipitates were analyzed by anti-phosphotyrosine (top). The membrane was stripped and reprobed with anti–TCR-ζ (middle) or with anti-STAT5 (bottom). No endogenous STAT5 was detected in these cells. (**C**) TCR-ζ facilitated STAT5 phosphorylation in cells expressing Lck. STAT5 was immunoprecipitated from lysates of 293T cells transfected with combinations of Lck, STAT5, and TCR-ζ expression vectors. Immunoprecipitates were subjected to protein immunoblotting with the monoclonal antibody 4G10 to phosphotyrosine (top). The blot was stripped and reprobed with anti-STAT5 (bottom). The ratio of cDNAs of Lck to STAT5A was 1:5 in lanes 1 and 4, 0.1:5 in lanes 2 and 5, and 0.01:5 in lanes 3 and 6. The ratio of cDNAs of TCR-ζ to STAT5A in lanes 4, 5, and 6 was 2:5. Empty vector DNA was used to adjust the amount of transfected DNA.

To confirm the association of STAT5 with the TCR upon T cell activation, we used T cells isolated from a TCR transgenic mouse specific for moth cytochrome C (MCC). With these cells, it was possible to induce TCR signaling by a combination of antigen-presenting cells (APCs) and an antigen peptide (pMCC) (*14*). $CD4^+$ T cells were mixed with APCs and either control peptide (pBSA) or antigen peptide (pMCC) for 5 min at 37°C. The TCR was immunoprecipitated with anti–CD3-ε as previously described (*14*), and the precipitates were analyzed for the presence of components of the TCR complex (Fig. 3A). STAT5 was more abundant in the receptor precipitate after antigen stimulation (Fig. 3A), indicating that the association of STAT5 with the TCR was induced by stimulation with the antigen peptide. Three repeated experiments confirmed the same results, and the TCR-ζ chain was also detected in the anti–CD3-ε precipitates in these experiments (*10*). In a complimentary experiment, STAT5 was immunoprecipitated from T cells treated with antigen or control peptide. The presence of the TCR-α chain was discovered in the precipitated complex only in samples from T cells stimulated with antigen peptide (Fig. 3B). Anti–nuclear factor kappa B (NF-κB) did not coimmunoprecipitate with the TCR complex (Fig. 3C). Taken together, these results suggest that STAT5 is specifically recruited to the TCR complex in response to T cell activation in vivo.

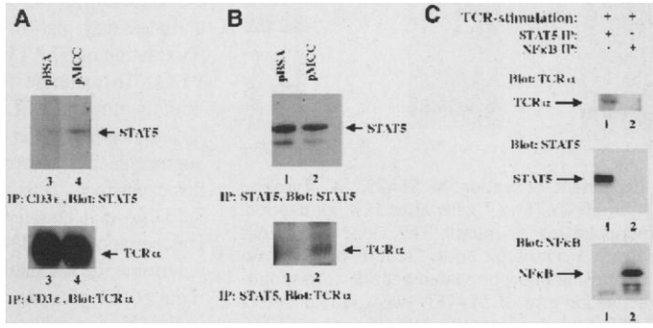

Fig. 3. STAT5 association and activation with the TCR complex during T cell activation in response to antigen stimulation. (**A**) STAT5-TCR interaction in mouse T cells carrying the MCC-specific T cell receptor. $CD4^+$ T cells from spleens and lymph nodes were cultured in the presence of specific cytokines to trigger either Th1 or Th2 differentiation. Cells were incubated with APCs that had been pulsed with agonistic peptide (pMCC) or inert peptide (pBSA). The TCR complex was immunoprecipitated from Brij cell lysates with anti–CD3-ε. Immunoprecipitates were separated by PAGE and transferred onto a membrane. Different regions of the membrane were analyzed for the presence of STAT5 (top) and TCR-α (bottom) by blotting with specific antibodies. (**B**) In the same manner as in (A), anti-STAT5 immunoprecipitates from murine $CD4^+$ T cells were analyzed. (**C**) STAT5-TCR interaction in D10 cells. D10 cells were treated with clonotypic anti-TCR and with secondary cross-linking antibody. Immunoprecipitations were carried out in Brij lysates with either agarose-conjugated anti-STAT5 or agarose-conjugated anti–NF-κB. Immunoprecipitates were analyzed for the presence of a TCR component by protein immunoblotting with anti–TCR-α (top). The presence of STAT5 and NF-κB in the respective immunoprecipitates was also shown (middle and bottom) by reprobing the membrane.

The presence of the TCR-α chain was discovered in the precipitated complex only in samples from T cells stimulated with antigen peptide (Fig. 3B). Anti–nuclear factor kappa B (NF-κB) did not coimmunoprecipitate with the TCR complex (Fig. 3C). Taken together, these results suggest that STAT5 is specifically recruited to the TCR complex in response to T cell activation in vivo.

D10 cells lack IL-2 production in response to TCR stimulation (*8*). We therefore could examine functions of STAT5 in TCR signaling without the possible complication of autocrine IL-2 stimulation. By stably expressing the epitope-tagged wild-type (FLAG-STAT5) or a dominant negative mutant (FLAG-Y694F) STAT5, we isolated variants of D10 cells, in which STAT5 activity was either enhanced (FLAG-STAT5) or inhibited (FLAG-Y694F) (Fig. 4A) (*15*). More colonies were formed in the wild-type STAT5 transfectants than in the mutant STAT5 transfectants (*10*). The proliferation of individual colonies was further measured in response to antigen stimulation (*16*). When stimulated by increasing doses of agonistic peptide, wild-type STAT5 transfectants displayed an enhanced proliferation rate compared with that of parental D10 cells (Fig. 4C). On the other hand, cells transfected with the dominant negative mutant STAT5 demonstrated a reduced response to antigen stimulation, resulting in decreased cell proliferation. Especially at low antigen doses (such as at the peptide concentration of 0.2 μg/ml), the response to antigen was inhibited in the STAT5 mutant (Fig. 4C). These results thus suggest that STAT5 is an important component of the TCR complex required for optimal sensitivity of the TCR to antigen stimulation for T cell proliferation. Furthermore, a functional STAT5 regulatory element was found in the promoter of the CD69 gene, and the immediate early expression of CD69 induced by TCR stimulation was diminished in D10 cells that were expressing the dominant negative STAT5 (STAT5-Y694F) (*10*), suggesting that STAT5 mediates TCR-induced transcriptional activation.

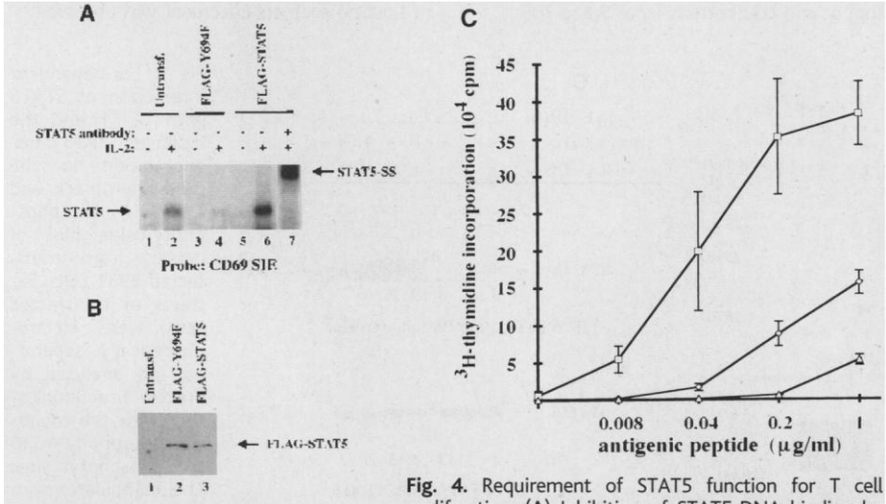

Fig. 4. Requirement of STAT5 function for T cell proliferation. (**A**) Inhibition of STAT5-DNA binding by FLAG STAT5 Y694F in a transfected D10 clone. Untransfected (Untransf.) D10 cells, FLAG-STAT5A wild-type transfectants, and FLAG-STAT5A Y694F transfectants were left untreated or treated with IL-2 (10 U/ml) for 15 min. IL-2–induced DNA binding of STAT5 was tested by EMSA. STAT5-SS: Antibody-induced supershift complex of STAT5 in lane 7. (**B**) Expression of FLAG-tagged STAT5 forms in D10 cells. FLAG-tagged STAT5A wild-type and STAT5A Y694F mutant were stably expressed in D10 cells. Clones were analyzed for the expression of transfected DNAs by protein immunoblotting with anti-FLAG. (**C**) Proliferation of D10 cells and STAT5 transfectants in response to antigen stimulation. Resting cultures of D10 cells (◇), FLAG-STAT5A wild-type transfectants (□), and FLAG-STAT5A Y694F transfectants (△) were incubated with APCs and different concentrations of agonistic peptide. Proliferation rates were determined 3 days later by measuring ^{3}H-thymidine incorporation into DNA. The results of a representative experiment with three samples per data ± SD are shown.

Studies of STAT5A/B double knockout mice also suggested a possible STAT5 func-

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.

References and Notes

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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).
10. T. Welte and X.-Y. Fu, unpublished results.
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 ($V_\alpha 14$ and $V_\beta 8$ 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 $V_\alpha 14^+$ 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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