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Enhanced Responses of Glycosylphosphatidylinositol Anchor-Deficient T Lymphocytes

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The functions of GPI-anchored proteins in T lymphocyte activation have been controversial. This issue was addressed by studying the responses of T lymphocytes from T lymphocyte-specific GPI anchor-deficient mice to different stimuli that normally allow coligation of TCR and GPI-anchored proteins. Stimulation of GPI anchor-deficient T lymphocytes with ConA induced 2-fold higher proliferative responses than did normal cells. In response to allogeneic stimulation, proliferation of GPI anchor-deficient T lymphocytes was enhanced 2- to 3-fold. The response to ConA of a GPI anchor-deficient anti-OVA T lymphocyte clone generated from these mice was ~3-fold higher than that of cells from the same clone in which GPI anchor expression was restored by retroviral transduction. The response of the GPI-anchor-deficient clone anti-OVA T lymphocytes to antigenic stimulation was similar to that of the retrovirally restored cells. These results indicate that coligation with GPI-anchored proteins counteracts the response to TCR stimulation by ConA or alloantigen but not protein Ag. The Journal of Immunology, 2004, 173: 3810–3815.

Materials and Methods

Generation of T lymphocyte-specific GPI anchor-deficient mice

T lymphocyte-specific Pig-a-deficient (LckCrePig-aΔloxP/loxP) mice were generated by Cre-loxP conditional targeting (reviewed in Ref. 14) as described (13). Briefly, female mice carrying loxP sites flanking exon 6 of Pig-a (15), an X-linked gene essential for the GPI anchor biosynthesis (3), were crossed with male mice expressing Cre recombinase driven by the T lymphocyte-specific promoter Lck (13). Both strains were kept in a C57BL/6 background.

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Abbreviations used in this paper: PNH, paroxysmal nocturnal hemoglobinuria; HSA, heat-stable Ag; CD24.

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6-129Sv genetic background. The presence of Cre and loxP and the disruption of Pig-a was detected in male offspring by PCR analysis as described (13, 15). DNA from mice positive for both Cre and loxP (LckCre/Pig-a-loxP-oxloxP mice) consistently demonstrated disruption of the Pig-a locus, and these male mice were used as T lymphocyte-specific Pig-a-targeted mice in experiments, at the age of 2-5 months. Age-matched male mice positive for loxP and negative for Cre (Pig-a-oxloxP mice) were used as littermate control mice. No differences were observed in numbers of splenocytes or lymph node cells isolated from LckCre/Pig-a-oxloxP mice or control mice. Mouse experiments have been approved by the institutional board.

Phenotypic analysis by flow cytometry

The expression of GPI-anchored proteins on different cell types was examined by flow cytometry using mAb against the GPI-anchored proteins Thy-1 (BD PharMingen, San Diego, CA), CD48 (ImmunoTech, Marseille, France), or HSA (BD PharMingen) and cell-specific markers. T lymphocytes were stained with mAb against the αβ TCR, CD4, CD8, CD45RA, or CD45RB (all from BD PharMingen); B cells were detected by mAb against B220 (BD PharMingen).

Stimulation of splenocytes with mitogen

Splenocytes were prepared by homogenization of spleens, hypotonic lysis of RBC, followed by two washes, and suspension in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (Life Technologies), 2 mM l-glutamine (Life Technologies), 35 μg/ml l-asparagine, 55 μM 2-ME, 100 U of penicillin/ml and 0.1 mg of streptomycin/ml (DMEM-10). Splenocytes from control or LckCre/Pig-a-loxP-oxloxP mice were seeded in 96-well tissue culture plates (Iwaki, Funahashi, Japan) at a concentration of 2 × 10^5 cells/well. ConA (Sigma-Aldrich) or PHA-P (PHA; Sigma-Aldrich) were added at various concentrations, and the cells were cultured for 48 h at 37°C in a humidified atmosphere. Eighteen hours before the end of the culture, 1 μCi of [3H]thymidine per well was added, and the radioactivity of the cells, determined by a scintillation counter, served as a measure for T cell proliferation.

Alloresponse

Lymph node (LN) CD4+ T lymphocytes were enriched from a pool of mesenteric, inguinal, axillary, and mandibular LN cells of LckCre/Pig-a-loxP-oxloxP, littermate control, or C57BL/6 mice by negative depletion using biotinylated Abs against anti-CD8 (BD PharMingen) and anti-I-Aβ (BD PharMingen) and magnetic MACS streptavidin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). This purification step typically generates cell populations containing >90% purified CD4+ cells as determined by flow cytometry, with no difference in yield between LckCre/Pig-a-oxloxP or control mice. Allogenic splenocytes were isolated from b6 mice (16) as described above, followed by irradiation with 2000 rad. To each well of 96-well tissue culture plates, 2 × 10^5 CD4+ LN cells and various numbers of irradiated b6 splenocytes were added. The cells were incubated for 5 days, 1 μCi of [3H]thymidine per well was added 18 h before the end of incubation, and the radioactivity of the cells was measured.

Generation of an anti-OVA T cell clone

Mice were immunized three times by i.p. injections with 50 μg of OVA with 4-wk interval. Serum samples were prepared at 3 wk after the first and at 1 and 3 wk after the second immunization. The total anti-OVA IgG levels in these sera were determined by ELISA as described (17). One week after the third immunization, splenocytes were isolated from an LckCre/Pig-a-oxloxP mouse as described above. Anti-OVA T lymphocytes were enriched and expanded by several restimulation cycles in the presence of 1 mg of OVA/ml, 10 U/ml rat rIL-2 (Genzyme-Techne, Cambridge, MA), and 4 × 10^5 irradiated (2000 rad) C57BL/6 splenocytes per well of 24-well tissue culture plates (Iwaki). Next, an anti-OVA T lymphocyte clone, designated 1D-OVA-A3, was generated by limiting dilution as described (18). This clone was maintained by culturing in the presence of IL-2, and repeated antigenic restimulation at intervals of ~2 wk and was CD4 positive by flow cytometry.

Restoration of Pig-a by retroviral transduction

The functional Pig-A cDNA was amplified from pEBP1G-Δ (3) as a template by PCR using the primer set of XhoF-PIGA (5'-GAATTCCCTCGAGTGGC CACCATGGCATCTCGAGGAGAGCTCGG and BamR-PIGA (5'-AAG CTGGATATCCGCGGCCTTACCTGGTTCCGATATCCCTT) to generate unique XhoI (5') and BamHI (3') sites and was digested by these two enzymes. After confirmation of the correct sequence, the plasmid was cloned between the XhoI and BamHI sites of the pRES2-EGFP vector (Clontech, Palo Alto, CA). This plasmid was digested by XhoI (5') and HpaI (3') to

FIGURE 1. Expression of GPI-anchored proteins on T and B lymphocytes from different organs. Expression of GPI-anchored proteins on T and B lymphocytes from spleens, lymph nodes, and peripheral blood was analyzed by flow cytometry. A, Splenic T lymphocytes stained with Ab against the T cell marker TCRβ and the GPI-anchored proteins Thy-1 (top panels) and CD48 (bottom panels). B, T lymphocytes from nonfractionated lymph nodes stained with Ab against TCRβ and Thy-1. C, T lymphocytes from peripheral blood stained with Ab against TCRβ and Thy-1. D, Splenic B lymphocytes stained with Ab against the B cell marker B220 and CD48; B220+ cells represent T lymphocytes. Left, cells from Pig-a-oxloxP mice (WT); right, cells from LckCre/Pig-a-oxloxP mice (knockout; KO); shown are whole lymphocyte populations gated in the forward/side-ward scatter.
FIGURE 2. Response of GPI-deficient T lymphocytes to mitogens. Splenocytes from male LckCre/Pig-d\textsuperscript{loxP}\ (knockout; KO; □), Pig-d\textsuperscript{loxP} littermate control (WT; △), or C57BL/6 mice (B6; △) were incubated with various concentrations of ConA (A) or PHA (B). T lymphocyte proliferative responses are expressed as uptake of [\textsuperscript{3}H]thymidine, shown are mean triplicate values ± SEM of at least three mice per group; * Significant differences (p < 0.05) with cells from both WT and B6 mice. In C and D, splenocytes from a female LckCre/Pig-d\textsuperscript{loxP} mouse with mosaic GPI-anchor-negative phenotype were incubated for 3 days with various concentrations of ConA (C) or PHA (D), and percentages of Thy-1\textsuperscript{−} T lymphocytes within the TCR\textsuperscript{αβ} population were assessed by flow cytometry. C and D. Percentages of Thy-1\textsuperscript{−} cells after incubation in medium reflect the percentages directly after isolation.

isolate a DNA fragment containing the Pig-A-IRES2-EGFP cDNA. The pMSCV-Pig-A vector plasmid was generated by cloning this fragment between the XhoI and HpaI sites of the retroviral vector plasmid pMSCVpuro (Clontech) (19), followed by digestion using NotI to eliminate the IRES2-EGFP fragment. Viral supernatant was prepared as described (20). Briefly, the amphotropic producer line AM12 was transfected with pMSCV-Pig-A, followed by infection of the ecotropic retroviral vector packaging cells E86 using AM12/pMSCV-Pig-A supernatant. The E86/pMSCV-Pig-A cells were grown to 80% confluence in DMEM-10, fresh medium was added, and flasks were incubated at 37°C for 16 h. The supernatants were collected, centrifuged to remove cell debris, aliquoted, and frozen at −70°C.

Next, in each well of a 24-well tissue culture plate precoated overnight with Retronectin (0.1 mg/ml; Takara Biomedicals, Otsu, Japan) and containing 5 × 10\textsuperscript{5} cells of the Pig-a-targeted T cell clone 1D-OVA-A3, a mixture of 5 × 10\textsuperscript{5} irradiated C57BL/6 splenocytes, 1 mg of OVA/ml, 20 U of IL-2/ml, and 50% (v/v) E86/pMSCV-Pig-A supernatant was added. After this infection cycle was repeated four times, ~27% of the cells expressed Thy-1 by flow cytometry. Thy-1\textsuperscript{−} cells were sorted by FACS and further expanded by repeated restimulation with OVA as described above. These cells retained Thy-1 expression for at least 2 months of culture.

For ConA or antigenic stimulation, 10\textsuperscript{5} of the original Pig-a-disrupted or Pig-a-restored 1D-OVA-A3 cells per well were incubated with ConA or OVA in the presence of 5 × 10\textsuperscript{5} irradiated C57BL/6 splenocytes for 48 h, and 1 μCi of [\textsuperscript{3}H]thymidine per well was present during the final 18 h. The radioactivity of the cells was measured to determine the proliferative response.

Results

GPI-deficient phenotype of T lymphocytes from LckCre/Pig-d\textsuperscript{loxP} mice

T lymphocyte-specific GPI-deficient mice (LckCre/Pig-d\textsuperscript{loxP}) were generated as described (13), by crossing mice carrying loxP-containing Pig-a with mice transgenic for the Cre recombinase driven by the T lymphocyte-specific promoter Lck. Consistent with previous observations (13), analysis by flow cytometry confirmed a lack of expression of the GPI-anchored proteins Thy-1 and CD48 on T lymphocytes in spleens (Fig. 1A), LN (Fig. 1B) or peripheral blood (Fig. 1C) from these mice. B lymphocytes in the spleens (Fig. 1D) or peripheral blood (not shown) expressed normal levels of the GPI-anchored proteins HSA (not shown) or CD48. These results confirm a clear GPI-deficient phenotype in T lymphocytes isolated from secondary organs from these mice.

Enhanced response of GPI anchor-deficient T lymphocytes to ConA

To investigate the role of GPI-anchored proteins in T cell activation, the proliferative response of GPI anchor-deficient T lymphocytes to various stimuli was studied. Splenocytes were stimulated with different concentrations of the mitogen ConA, and the T lymphocyte proliferative response was determined. At ConA concentrations of 2 μg/ml or lower, the proliferative response of GPI-deficient T lymphocytes as assessed by [\textsuperscript{3}H]thymidine uptake was significantly enhanced, being ~2-fold higher than that of control T lymphocytes (Fig. 2A).

The mean percentages of T lymphocytes relative to the total splenocyte populations ± SEM were similar, being 36.0 ± 3.4 for LckCre/Pig-d\textsuperscript{loxP}, 37.5 ± 0.7 for Pig-d\textsuperscript{loxP}, and 34.8 ± 1.3 for C57BL/6 mice (n = 5). Thus, the difference in the response to
Enhanced responses to ConA, the proliferative response of GPI anchor-deficient splenocytes to various concentrations of PHA was similar to that of control cells (Fig. 2B).

These results were confirmed using splenocytes from female LckCre/Pig-a<sup>ox</sup> mice, which have a mosaic GPI anchor-negative phenotype due to X-chromosome inactivation. After incubation with ConA, both the total number of cells (not shown) and percentage of Thy-1<sup>-</sup> T cells (Fig. 2C) had increased, indicating that GPI-deficient cells had proliferated more. In contrast, after incubation with PHA, the total number of cells had increased but the percentage of Thy-1<sup>-</sup> cells remained unchanged (Fig. 2D), indicating that GPI anchor-negative and -positive cells had proliferated similarly. Thus, the absence of the GPI anchor confers on T lymphocytes an enhanced responsiveness to low concentrations of ConA.

Enhanced alloreactivity by GPI anchor-deficient T lymphocytes

Next, the response of GPI-deficient T lymphocytes to allogeneic stimulation was studied. Purified CD4<sup>+</sup> T lymphocytes from lymph nodes were incubated in the presence of irradiated allogeneic splenocytes from bm12 mice, which are recognized as alloantigen by CD4<sup>+</sup> T lymphocytes from mice in a C57BL/6 genetic background due to a point mutation in IA<sup>b</sup> (16). When stimulated with allogeneic cells, the proliferative response of GPI anchor-deficient T lymphocytes was 2- to 3-fold higher than that of control T lymphocytes (Fig. 3). The difference in response between GPI-deficient T lymphocytes and control cells was not apparent when stimulated with a high concentration of allogeneic cells (Fig. 3). Thus, T lymphocytes lacking the GPI anchor are hyperresponsive to allogeneic stimulation.

Responses of a GPI anchor-deficient anti-OVA T lymphocyte clone

To generate an anti-OVA-specific T lymphocyte clone, mice were immunized three times with OVA. No differences in total anti-OVA IgG levels in sera of control or LckCre/Pig-a<sup>ox</sup> mice were observed, either at 3 wk after the first or 3 wk after the second immunization (not shown). One week after the third immunization, splenocytes were isolated. The proliferative responses of splenocytes freshly isolated from LckCre/Pig-a<sup>ox</sup> control mice to intravenous restimulation with OVA were not different (not shown). A T lymphocyte clone (1D-OVA-A3) specific for OVA was generated from LckCre/Pig-a<sup>ox</sup> splenocytes by limiting dilution. Analysis by flow cytometry demonstrated that this clone was CD4<sup>+</sup> (not shown) and did not express Thy-1 (Fig. 4, left), confirming its GPI anchor-negative phenotype. Retroviral transduction with MSCV-PIG-A restored Thy-1 expression in ~27% of these cells (Fig. 4, middle). Parallel experiments using MSCV-PIG-A showed restoration of Thy-1 expression in ~45% of BWS147 Thy-1<sup>-</sup> cells, a Pig-a-deficient T lymphoma cell line (22) (not shown). Next,
A previous study showed normal ConA responses of GPI anchor-deficient T lymphocytes, using an MTT assay (13) which determines the amount of a mitochondrial enzyme present in the cells. In the present study, the proliferative response was assessed either by [3H]thymidine uptake as a measure for DNA replication or by cell numbers. The reason for the difference in the previous and present results is likely a different sensitivity in detecting the initiation of a proliferative response. In another study, GPI-negative T cells from PNH patients were shown to exhibit normal responses to CD3 stimulation and decreased response to PHA (28). The difference between the latter finding and the normal PHA responses by murine GPI-deficient T cells observed in the present study may be related to different affinities of PHA for its ligands on murine or human cells, in addition to the emerging evidence that cells from PNH patients have genetic changes other than GPI deficiency alone (29).

Discussion

This study shows that GPI-anchor deficiency leads to strongly enhanced responses of T lymphocytes to ConA or allogeneic stimulation. Cells from the Pig-a-deficient anti-OVA T cell clone 1D-OVA-A3 (knockout; KO), or cells from the same clone after full Pig-a restoration (KO + A) were stimulated with ConA (A) or with OVA (B), in the presence of APCs. T lymphocyte proliferative responses are expressed as uptake of [3H]thymidine; shown are mean triplicate values ± SEM of a representative experiment of three.

Thy-1+ 1D-OVA-A3 cells were isolated by FACS and further expanded. More than 99% of these sorted cells retained full Thy-1 positivity during expansion for at least 2 months (Fig. 4, right).

The original GPI anchor-deficient 1D-OVA-A3 clone and the retrovirally Pig-a restored clone allowed functional analysis of GPl anchor deficiency by comparison between these otherwise genetically identical clones. The proliferative responses of the original GPl-deficient clone to stimulation with ConA was ~2-fold higher than that of the Pig-a restored clone (Fig. 5A). This is in agreement with the enhanced response of splenocytes from Lck-Cre/Pig-dox mice (Fig. 2A). After antigenic stimulation with OVA and irradiated splenocytes as APCs, the response of GPl-deficient 1D-OVA-A3 T lymphocytes was indistinguishable from that of Pig-a restored cells (Fig. 5B). Similar results were obtained when titrating APCs with a fixed concentration of OVA, or when using irradiated purified splenic C57BL/6 dendritic cells as APCs instead of irradiated total splenocyte populations (not shown). These results indicate that GPl anchor deficiency causes enhanced responses of the anti-OVA T lymphocyte clone to ConA, but not to stimulation with protein Ag.

The response to stimulation of the anti-OVA T cell clone by protein Ag was not affected by the absence of the GPl anchor. The reason why at this condition, TCR triggering could not efficiently be suppressed by coligation of GPl-anchored proteins, may lie in a relatively strong TCR stimulus, possibly caused by a high affinity for peptide-MHC. A strong TCR stimulus may overcome the inhibitory effect of the GPl-anchored proteins. The balance between TCR-mediated stimulation and GPl anchor-mediated suppression would thus determine the outcome.

The mechanism for the inhibitory activity of coligation of TCR and GPl-anchored proteins remains to be clarified. GPl-anchored proteins may indirectly inhibit T cell signaling through their interaction with other inhibitory molecules. In this respect, it has been shown that the GPl-anchored protein Thy-1 is closely associated with CD45 (31), a protein tyrosine phosphatase capable of regulating T cell activation (32). An alternative explanation may come from the lipid raft hypothesis (33, 34). Plasma membranes contain specialized lipid microdomains or lipid rafts that concentrate GPl-anchored proteins at the outer leaflet and cytosolic tyrosine kinases at the inner leaflet (35). Activation of T cells through TCR has been shown to involve redistribution of TCR into these rafts (36, 37). Recently, we found that ligand-induced effector functions of IgG-Fc receptors, which also use lipid rafts, are defective in the absence of the GPl anchor (38). It is possible that the absence of GPl-anchored proteins disturbs TCR-raft association, leading to abnormal responses to TCR stimulation. Further insight into the molecular and functional interactions between GPl-anchored proteins and the TCR or its signaling components will deepen our understanding of this inhibitory mechanism. Negative regulation of T cell responses by GPl-anchored proteins may be relevant for and constitutively operative during normal immune responses, T cell development, or the control of autoimmunity.
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