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Lysosomal Glycosphingolipid Recognition by NKT Cells

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NKT cells represent a distinct lineage of T cells that coexpress a conserved αβ T cell receptor (TCR) and natural killer (NK) receptors. Although the TCR of NKT cells is characteristically autoreactive to CD1d, a lipid-presenting molecule, endogenous ligands for these cells have not been identified. We show that a lysosomal glycosphingolipid of previously unknown function, isoglobotrihexosylceramide (iGb3), is recognized both by mouse and human NKT cells. Impaired generation of lysosomal iGb3 in mice lacking β-hexosaminidase b results in severe NKT cell deficiency, suggesting that this lipid also mediates development of NKT cells in the mouse. We suggest that expression of iGb3 in peripheral tissues may be involved in controlling NKT cell responses to infections and malignancy and in autoimmunity.

As with protein-derived antigens, lipids, glycolipids, and lipopeptides (either of microbial or self origin) can be recognized by TCRαβ-expressing T lymphocytes (*1*). Of these, NKT cells represent an unusual population that recognizes lipids presented by the MHC class I–like CD1d protein and displays characteristics of innate rather than adaptive lymphocytes (*2*). The TCR of NKT cells is limited mainly to a single invariant α chain (mouse Vα14-Jα18 and the homologous human Vα24-Jα18) combined with variable mouse Vβ8 and human Vβ11 TCR β. These cells express a phenotype of effector or memory lymphocytes before encounter with any foreign antigen and display a panoply of inhibitory receptors also expressed on NK cells. Such features suggest that they may respond to conserved endogenous ligands, as well as foreign microbial antigens (*3*).

Mouse (m) Vα14 and human (h) Vα24 NKT cells appear to regulate a number of conditions in vivo, including malignancy and infection, as well as autoimmune diseases, through the rapid secretion of T helper 1 (T_H1) and T_H2 cytokines and chemokines (*4*). Without knowledge of the natural antigens recognized by these cells, it has been difficult to explore the mechanisms that govern their recruitment, activation, and development. Previous work has established the requirement for lysosomal trafficking of CD1d molecules (*5*) and the role of lysosomal proteases in presenting endogenous lipid antigens (*6*); the essential function of lysosomal lipid transfer proteins, known as sphingolipid activator proteins, or saposins, is now also established (*7–9*). These findings, and the recent report that a β-glucosylceramide synthase mutant cell line was defective in Vα14 NKT cell stimulation (*10*), have indicated that the natural ligands of NKT cells might be lysosomal glycosphingolipids.

We found that mice genetically deficient in the lysosomal glycosphingolipid degrading enzyme β-hexosaminidase b subunit ($Hexb^{-/-}$) (*11–13*) exhibited a severe reduction in the number of Vα14 NKT cells (Fig. 1, A and B). Thus, staining for NKT cells in these mice using tetramers of CD1d complexed with the artificial lipid αGalCer (CD1d-αGalCer) was reduced by 95% on average. All subsets of NKT cells, including the earliest $CD44^{low}NK1.1^{-}$ precursor and the CD4 and $CD4^{-}8^{-}$ cells, were equally affected as early as these cells could be detected in young 2.5-week-old mice [fig. S1 and (*14*)]. In contrast, the development of classical, naïve, and memory CD4 and CD8 T cells, as well as B cells, γδ T cells, and NK cells were not affected by Hexb deficiency [Fig. 1D and (*14*)].

Although CD1d surface expression was unaltered in $Hexb^{-/-}$ mice (Fig. 1C), thymocytes from these animals failed to elicit a response from a $Vα14^{+}$ NKT cell hybridoma (DN32.D3) (Fig. 2A). In contrast, they normally stimulated a $Vα14^{-}$, CD1d-reactive NKT hybridoma (TCB11) (Fig. 2A). Presentation of the ligand of DN32.D3, but not that of TCB11, is dependent on lysosomal function (*7*); these results suggested defects in presentation of lysosomal ligands. To rule out general, nonspecific lysosomal defects, resulting, for example, from lysosomal lipid storage in these mutant mice, we tested the lysosomal functions of $Hexb^{-/-}$ cells using a panel of diglycosylated αGalCer derivatives that require lysosomal processing before recognition by Vα14 NKT cells (*15*). The presentation of *N*-acetylgalactosamine (GalNAc) β1,4 Gal αCer by cells deficient in β-hexosaminidase b was selectively defective (Fig. 2B, upper panel), as expected from the specificity of this enzyme (*11–13*). In contrast, α-galactosidase A (αGalA)–deficient cells were selectively defective in processing Gal α1,4 Gal αCer and Gal α1,2 Gal αCer (Fig. 2B, lower panels), as expected from the specificity of αGalA (*15*). In contrast to these specific processing defects, control cells expressing a truncated form of CD1d missing the cytoplasmic endosomal targeting [CD1-TD "knock-in" (*5*)] were impaired in the presenta-

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tion of all the diglycosylated αGalCer precursors we tested (Fig. 2B). Thus, Hexb$^{-/-}$ cells had a highly specific defect in the generation of the lysosomal ligands of Vα14 NKT cells.

Therefore, one of the natural products of the Hexb-dependent enzymes (β-hexosaminidase A and B) may be the ligand of mVα14 NKT cells. In mammalian cells, these two enzymes remove β-linked GalNAc residues found on glycosphingolipids of the ganglio-series (GalNAc β1,4 Gal) and the globo- and isoglobo-series (GalNAc β1,3 Gal), as well as GlcNAc residues of the lacto-series (GlcNAc β1,3 Gal) (*16*). Mice lacking ganglio-series glycolipids as a result of genetic deficiency of the key enzymes responsible for their synthesis, GM2 synthase and GM3 synthase (*17*, *18*), displayed no apparent defect in NKT cell development (Fig. 1B). Focusing on the other three series, we chemically synthesized globotrihexosylceramide Gb3 (Gal α1,4 Gal β1,4 Glc β 1,1 Cer) and isoglobotrihexosylceramide iGb3 (Gal α1,3 Gal β1,4 Glc β 1,1 Cer) (fig. S3), and purified the lacto-series glycolipid Gal α1,3 Gal β1,4 GlcNAc β 1,3 Gal β1,4 Glc β 1,1 Cer (*19*) to test their ability to stimulate NKT cells. Of these, only iGb3 displayed stimulatory activity [Fig. 3 and (*14*)]. Thus, iGb3 selectively expanded human Vα24 NKT cells in 4-day cultures of fresh peripheral blood mononuclear cells (PBMCs) (Fig. 3B) and stimulated strong T_H1 [as measured by interferon-γ (IFN-γ)] and T_H2 [as measured by interleukin 4 (IL-4)] cytokine secretion by a polyclonal human Vα24 NKT line (Fig. 3C). Fluorescence-activated cell sorting (FACS) analysis for intracellular IFN-γ revealed that about 50% of the cells stimulated by αGalCer responded to iGb3 [fig. S2A (*20*)], although this is likely an underestimate, because iGb3 elicited less IFN-γ per cell. Both of two NKT cell subclones tested, one with a CD4$^+$ and the other a CD4$^-$8$^-$ phenotype, also responded to iGb3 (*14*). iGb3 derived from other sources, including natural iGb3 purified from cat intestine (*19*) and iGb3 produced in vitro by action of iGb3 synthase on lactosylceramide and uridine diphosphate (UDP)–galactose, (fig. S3) were as stimulatory as synthetic iGb3 (Fig. 3C, right panel; fig. S3). iGb3 presented by CD1d-expressing bone marrow–derived dendritic cells stimulated the mVα14 NKT cell hybridoma DN32.D3 (Fig. 3D) and all the other mVα14 hybridomas tested, whether they used TCR Vβ8, Vβ7, or Vβ2, although responses were not elicited from any of the non-Vα14 hybridomas (fig. S2B). No response was observed with CD1d$^{-/-}$ dendritic cells (*14*).

In the lysosome, β-hexosaminidase removes the terminal GalNAc of iGb4 to produce iGb3, with α-galactosidase A subsequently transforming iGb3 into lactosylceramide (LacCer) by removal of the terminal Gal (see Fig. 3A). iGb4 presented by bone marrow–derived dendritic cells was stimulatory for mouse (Fig. 3D, right panel) and human NKT cells (*14*), whereas LacCer was not (Fig. 3C). However, Hexb$^{-/-}$ cells presented iGb3 but failed to present iGb4, which indicated directly that processing of iGb4 into iGb3 is necessary for NKT cell recognition (Fig. 3D). Trafficking of CD1d to lysosomal compartments was essential to present these antigens, as shown using cytoplasmic tail–truncated CD1d (CD1-TD)–expressing antigen-presenting cells (APCs) (Fig. 3D), and the absence of lysosomal saposins impaired iGb3 presentation (Fig. 3E). These results are consistent with previous reports showing that NKT cell development and function required CD1d trafficking to lysosomal compartments (*5*), as well as the lipid transfer function of saposins (*7*).

In a cell-free assay (*7*, *21*), loading of iGb3 and iGb4 onto CD1d required saposin B (Fig. 3F, left panel). Attempts to stain NKT cells directly with CD1d/iGb3 tetramers were unsuccessful, perhaps because of a low affinity for TCR and our inability to achieve 100% loading with iGb3 even in the presence of saposins (see Fig. 3F). Nevertheless, we found that CD1d/iGb3, but not CD1d/iGb4, complexes could significantly

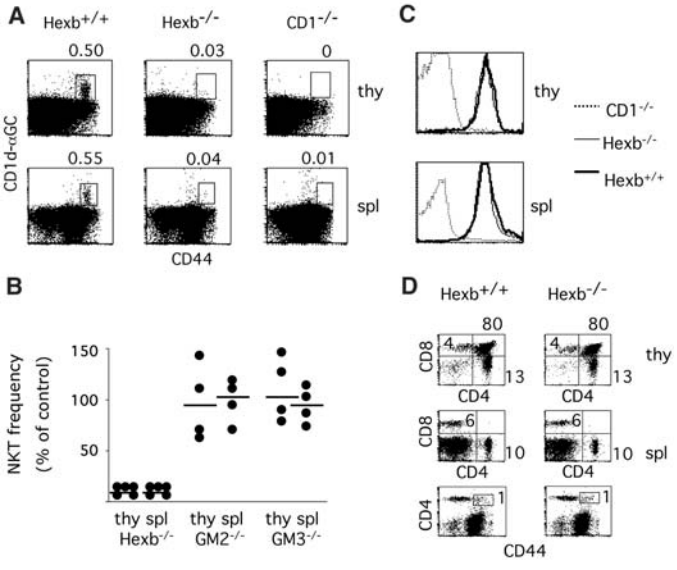

Fig. 1. Deficient thymic selection of Vα14 NKT cells in Hexb$^{-/-}$ mice. (**A**) Lymphocytes from thymus and spleen of Hexb$^{+/+}$ and Hexb$^{-/-}$ littermates, and CD1d$^{-/-}$ mice were stained with CD1d-αGalCer tetramers and anti-CD44. Representative FACS profiles are shown with percentages indicated in the upper quadrants. Absolute numbers of lymphocytes in the thymus and the spleen of mutant and wild-type mice were similar. The NKT cell defect was also observed in the liver (*14*). (**B**) Summary of experiments where the frequency of thymic (thy) and splenic (spl) NKT cells in individual mutant mouse is represented as a percentage of control wild-type littermate. GM2$^{-/-}$ and GM3$^{-/-}$ mice are lacking GM2 synthase and GM3 synthase, respectively. (**C**) FACS analysis of CD1d in thymocytes and splenocytes. (**D**). CD4/CD8 and CD4/CD44 profiles of thymocytes and splenocytes.

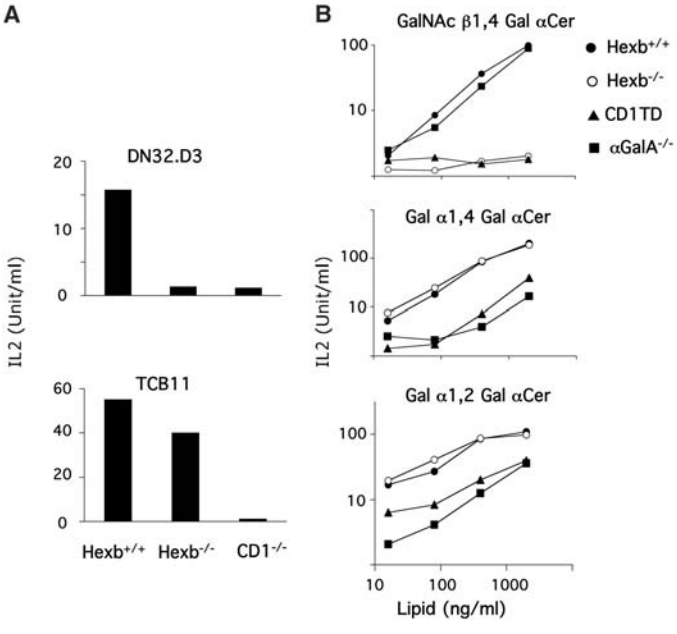

Fig. 2. Specific antigen presentation defects in Hexb$^{-/-}$ mice. (**A**) Autoreactive responses of Vα14$^+$ DN32.D3 and Vα14$^-$ TCB11 hybridomas stimulated by CD1d-expressing thymocytes from Hexb$^{-/-}$ and Hexb$^{+/+}$ littermates. CD1$^{-/-}$ thymocytes served as negative control. (**B**) Spleen cells from Hexb$^{-/-}$ and Hexb$^{+/+}$ littermates, αGalA$^{-/-}$, and CD1-TD knock-in mice were pulsed with various diglycosylated variants of αGalCer, as indicated, before stimulation of Vα14$^+$ DN32.D3 hybridoma. The data are representative of two separate experiments.

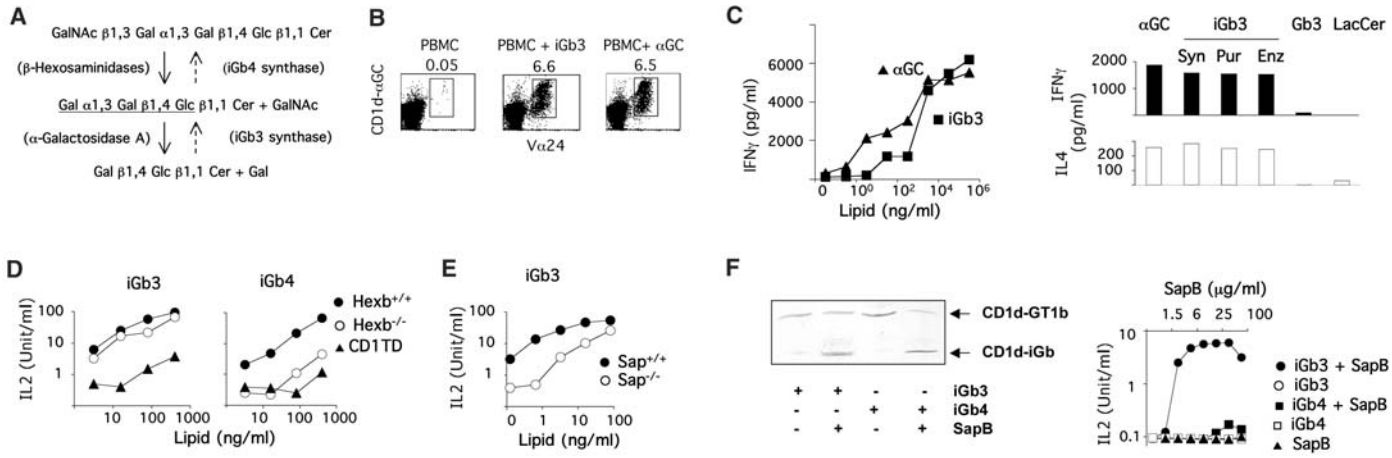

Fig. 3. iGb3 is the ligand of mVα14 and hVα24 NKT cells. (**A**) A schematic of synthesis (dotted arrows, right) in the Golgi, and degradation (continuous arrows, left) in the lysosome, of iGb3. From top to bottom, iGb4, iGb3, and LacCer. (**B**) Frequency of hVα24 NKT PBL, doubly-stained by antibody against Vα24 and CD1d-αGalCer tetramers, in PBMCs cultured for 4 days in the presence of 100 ng/ml αGalCer, iGb3, or medium alone, as indicated. Similar results were found in six out of six healthy human subjects. (**C**) (Left) IFN-γ production by a human Vα24 NKT line stimulated with different concentrations of iGb3 and αGalCer in the presence of irradiated PBMCs as CD1d-expressing APCs. Similar results were obtained with two out of two additional cloned CD4 and DN Vα24 NKT lines. (Right) IFN-γ and IL-4 production by the human Vα24 NKT line in response to irradiated PBMCs and 100 ng/ml of iGb3 of synthetic, purified, and enzymatic origin, versus 100 ng/ml of αGalCer, Gb3, or LacCer, as indicated. Similar results were obtained with two out of two additional CD4 and CD4$^-$CD8$^-$ hVα24 NKT clones. (**D**) Stimulation of mouse Vα14$^+$ DN32.D3 by iGb3 and iGb4 presented by bone marrow–derived dendritic cells from Hexb$^{-/-}$, Hexb$^{+/+}$, and CD1-TD mice as indicated. (**E**) Stimulation of mouse DN32.D3 by iGb3 with bone marrow–derived dendritic cells from saposin-deficient (Sap$^{-/-}$) and -sufficient (Sap$^{+/+}$) littermates, as indicated. (**F**) (Left) In vitro loading of iGb3 and iGb4 onto recombinant CD1d in the presence of saposin B, visualized by isoelectrofocusing. Electromobility shift indicates partial replacement of GT1b by iGb3 and iGb4, as indicated. (Right) Stimulation of DN32.D3 by iGb3 and iGb4 loaded on plate-bound CD1d in the presence of saposin B, as indicated.

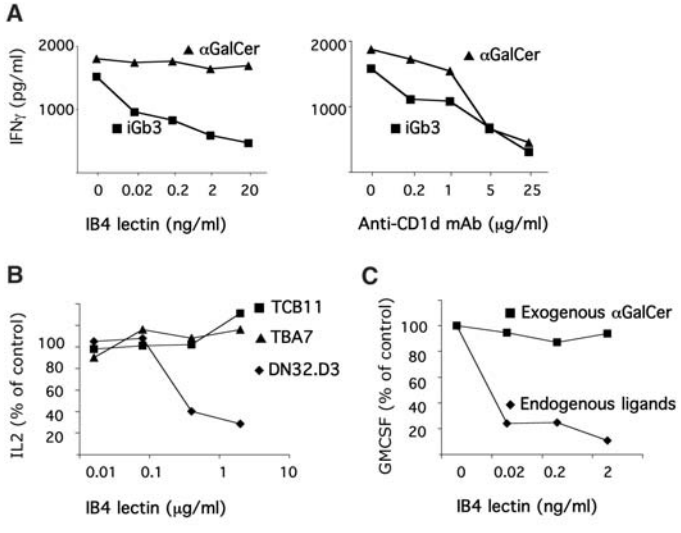

Fig. 4. Blocking CD1d/iGb3 stimulation by the lectin IB4. (**A**) IB4 inhibited the stimulation of the human Vα24 NKT line by iGb3 but not αGalCer pulsed PBMC (left). In contrast, anti-human CD1d mAb 51 inhibited stimulation by both iGb3 and αGalCer (right). (**B**) IB4 specifically inhibited the CD1d-autoreactive response of Vα14$^+$ DN32.D3 but not that of non-Vα14 hybridomas TCB11 and TBA7 to RBL.CD1d. Results expressed as a percentage of control without lectin and representative of four separate experiments. (**C**) IB4 inhibited the CD1d-autoreactive response of the hVα24 NKT line to PBMC-derived dendritic cells alone (endogenous ligands), measured by enzyme-linked immunosorbent assay (ELISA) as granulocyte/macrophage colony-stimulating factor (GMCSF) released in the supernatant. The control response to PBMC-derived dendritic cells plus exogenous αGalCer was not altered. Results are expressed as a percentage of control without lectin (i.e., 939 pg/ml for exogenous ligand and 294 pg/ml for endogenous ligand) and are representative of three separate experiments.

stimulate Vα14 NKT cells (Fig. 3F, right panel). These results reveal that, whereas both iGb3 and iGb4 can bind CD1d, only iGb3 is a major antigen of the Vα14 NKT cell and that removal of the distal GalNAc residue of its precursor iGb4 by lysosomal β-hexosaminidase is required for TCR recognition in vivo.

The almost-complete block in Vα14 NKT cell development in Hexb$^{-/-}$ mice and the inability of Hexb$^{-/-}$ thymocytes to stimulate Vα14 NKT hybridomas suggested that iGb3 might alone represent the principal natural ligand of NKT cells. To test this further, the *Griffonia simplicifolia*–derived isolectin B4 (IB4), a lectin that binds the terminal Gal α1,3 Gal of iGb3 (*22*), was used to inhibit NKT cell stimulation. IB4 impaired hVα24 NKT cell stimulation by exogenously added iGb3, but not by αGalCer (Fig. 4A, left panel), whereas a monoclonal antibody (mAb) against CD1d blocked stimulation by both glycolipids (Fig. 4A, right panel). These results are consistent with specific recognition by IB4 of the terminal carbohydrates of iGb3, even when bound to CD1d. This binding property of IB4 was next exploited to test whether these terminal Gal α1,3 Gal residues contribute significantly to the natural stimulation of mVα14 and hVα24 NKT cells. IB4 prevented the autoreactive stimulation of the Vα14$^+$ DN32.D3 hybridoma by rat basophilic leukemia (RBL) cells transfected to express mouse CD1d (Fig. 4B). In contrast, stimulation of two control (non-Vα14) CD1d autoreactive hybridomas was unaffected (Fig. 4B). Furthermore, IB4 also blocked natural recognition of CD1d-expressing PBMC-derived dendritic cells by the human Vα24 NKT line but failed to block recognition of exogenously added αGalCer (Fig. 4C). Blockade of iGb3 recognition by IB4 in humans consistently required lower amounts of lectin (by a factor of ~1000) than for mouse and rat cells, possibly because mice and rats, but not humans, express abundant levels of an additional ligand recognized by IB4, the Gal α1,3 Gal epitope expressed on glycoproteins (*23*), which would compete for binding.

Because of their role in regulating a range of disease states, the nature of ligands recognized by mVα14/hVα24 NKT cells has been the subject of intense research and speculation (*24*–*26*). Our findings suggest that a single glycosphingolipid, the isoglobo-side iGb3, may represent the principal endogenous ligand of mVα14 and hVα24 NKT cells, at least under conditions not associated with disease. It should be noted, however, that glycosphingolipids of the isoglobo-series have not been purified and characterized in all mammalian species yet. In particular, while they have been biochemically demonstrated in rat (*22*), dog, and cat (*27*), they have not been reported in mouse and human. Both mouse and human, however, have the iGb3 synthase gene, which we showed to be expressed and functional in mouse (fig. S3, A to C). Furthermore, the Hexb requirement for natural ligand expression in mouse and the blockade of its recognition by IB4 in mouse and human suggest that this natural ligand must have a sequence of carbohydrates identical or highly similar to that of iGb3, i.e., a [Gal α1,3 Gal] exposed on removal of a hexosamine in the lysosome. In humans, natural antibodies against [Gal α1,3 Gal] epitopes have been reported (*23*), raising the possibility that they might interfere with iGb3 or that iGb3 might be low or absent. We have shown that these natural antibodies against Gal do not recognize iGb3 [fig. S4 and (*27*, *28*)]. Thus, despite the current lack of direct biochemical evidence for the presence of iGb3 in mouse and human, the combined data suggest that iGb3 or a close structural analog is the principal self antigen of NKT cells. Our results, however, do not rule out the existence of additional endogenous ligands. For example, the few residual Vα14 NKT cells found in $Hexb^{-/-}$ mice might recognize other ligands. It is also possible that alternative endogenous ligands are expressed in some disease conditions or in particular cell types not examined here. Direct biochemical studies will be required to elucidate these issues.

The lack of NKT cell precursors in the thymus of $Hexb^{-/-}$ mice suggests that iGb3 is also the ligand involved in their thymic development. Although this hypothesis remains to be confirmed, it is consistent with the model that the unusual effector memory phenotype imparted to the thymocyte precursors of NKT cells is a consequence of their thymic stimulation by agonist ligands, i.e., antigens that also stimulate the mature NKT cell (*29*, *30*). Our findings should allow a further dissection of the mechanisms underlying the development of these autoreactive, regulatory lymphocytes.

It is possible that lysosomal expression of iGb3 is dysregulated in diseases regulated by NKT cells, such as type I diabetes and cancer (*4*, *31*) or microbial infection (*32*). Additional ligands, such as the mycobacterial glycolipid phosphatidylinositolmannoside (*33*), may also elicit NKT cell responses. Thus, the discovery of natural endogenous and microbial NKT cell ligands, as well as the synthesis of pharmacologic agonists or inhibitors, may lead to novel approaches to manipulating NKT cells for the prevention and treatment of diseases.

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Crystal Structure of a Photolyase Bound to a CPD-Like DNA Lesion After in Situ Repair

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DNA photolyases use light energy to repair DNA that comprises ultraviolet-induced lesions such as the *cis-syn* cyclobutane pyrimidine dimers (CPDs). Here we report the crystal structure of a DNA photolyase bound to duplex DNA that is bent by 50° and comprises a synthetic CPD lesion. This CPD lesion is flipped into the active site and split there into two thymines by synchrotron radiation at 100 K. Although photolyases catalyze blue light–driven CPD cleavage only above 200 K, this structure apparently mimics a structural substate during light-driven DNA repair in which back-flipping of the thymines into duplex DNA has not yet taken place.

Life under the sun is endangered by ultraviolet (UV) radiation that causes the formation of genotoxic photoproducts in DNA (*1*). Major UV-induced lesions include *cis-syn* cyclobutane pyrimidine dimers (CPDs) formed by a [2+2] cycloaddition of two adjacent pyrimidine bases, usually thymine. The importance of efficient repair systems for UV lesions is highlighted by hereditary diseases such as xeroderma pigmentosum. In prokaryotes, plants, and many animals, DNA photolyases (EC no. 4.1.99.3) are mainly responsible for the repair of CPD lesions by catalyzing the cleavage of the cyclobutane ring, using blue or near-UV light [absorbance (λ) of 360 to 500 nm] as the energy source (*2*, *3*).