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REVIEW

Immunological Functions of Steryl Glycosides

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Abstract Steryl glycosides, sterols glycosylated at the 3β-hydroxy group, have been widely found in plants, algae, and fungi, but are rare in bacteria and animals. Glycosylation of sterols is known to modify properties of the cell membrane and confer resistance against stresses by freezing or heat-shock on cells. Furthermore, accumulating evidence obtained from recent research suggests important biological functions of steryl glycosides, including regulation of host defenses against pathogens, lipid metabolism, and developmental events. This review is focused on the immunological functions of steryl glycosides, such as modulation of host immune functions upon exposure to cholesteryl glycosides produced by pathogenic bacteria.

Keywords Steryl glycoside · Invariant NKT cell · *Helicobacter pylori* · Immunomodulation

Abbreviations

ChG	Cholesteryl glucoside
ChAcG	Cholesteryl 6′-*O*-acyl α-glucopyranoside
ChPG	Cholesteryl 6′-*O*-phosphatidyl α-glucopyranoside
SG	Steryl glycoside
*i*NKT	Cell invariant Vα14-Jα18 TCR α-bearing NKT cell

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Introduction

Steryl glycosides (SGs) are derivatives of sterols (steroids with a hydroxy group at the C3 carbon in ring A of four condensed aliphatic rings), where the sugar moiety is attached to the 3-hydroxy group.

SG was first isolated from olives in 1908 (Power and Tutin 1908) and this compound was later identified as sitosteryl D-glucoside (Power and Salway 1913; Salway 1913). Phytosteryl 6′-*O*-acyl-glucoside, an esterified form of SG, was then isolated from soybeans and potatoes (Lepage 1964). SGs and their acylated form are ubiquitously present in vascular plants. They are also abundant in many kinds of fungi and algae, but have been relatively rarely found in bacteria. Comprehensive reviews of the structure, distribution, biosynthesis of SGs have been published (Kovganko and Kashkan 1999; Grill et al. 2010).

In contrast to the ubiquitous occurrence of steryl glycosides in plants and fungi, knowledge regarding the presence of SGs in animals is limited (Kovganko and Kashkan 1999; Grill et al. 2010); cholesteryl β-glucoside was found in the skin of snakes (Abraham et al. 1987), birds (Wertz et al. 1986) and human fibroblast cell lines (Kunimoto et al. 2000; Kunimoto et al. 2002), while cholesteryl β-glucopyranuronic acid was isolated from the human liver (Hara and Taketomi 1982; Muhiudeen et al. 1984; Taketomi et al. 1982). However, intracellular localization of steryl glycosides in animal cells has not been elucidated. Furthermore, since cloning of the genes responsible for sterol glycosyltransferase has not yet been accomplished for animals, a genetic approach was not applicable to the functional characterization of SGs. Thus, the biological significance of endogenously synthesized SGs in animals, which has not yet been characterized, is out of the range of this review.

In the present review, attention is focused on the immunological functions of SGs that have been obtained from observations when subjects are exposed to exogenously administrated SGs or when they interact with pathogenic bacteria producing SGs.

Structure and Distribution of Steryl Glycosides

Steryl glycosides contain structural variations both in the sterol and sugar moiety (Fig. 1).

Sterol Heterogeneity

The composition of sterols in SGs reflects the content of free sterols. SGs in plants consist of sitosterol, while most SGs in fungi possess the ergosterol moiety. SGs synthesized in animals consist of cholesterol. SGs found in certain auxotrophic bacteria, such as *Helicobacter pylori* (Hirai et al. 1995), contain cholesterol. It is believed that the cholesterol present in such pathogenic bacteria arises from the host cells (Wunder et al. 2006).

The configuration of the 3-hydroxy group in SGs is usually β (extruding from the planar condensed rings)

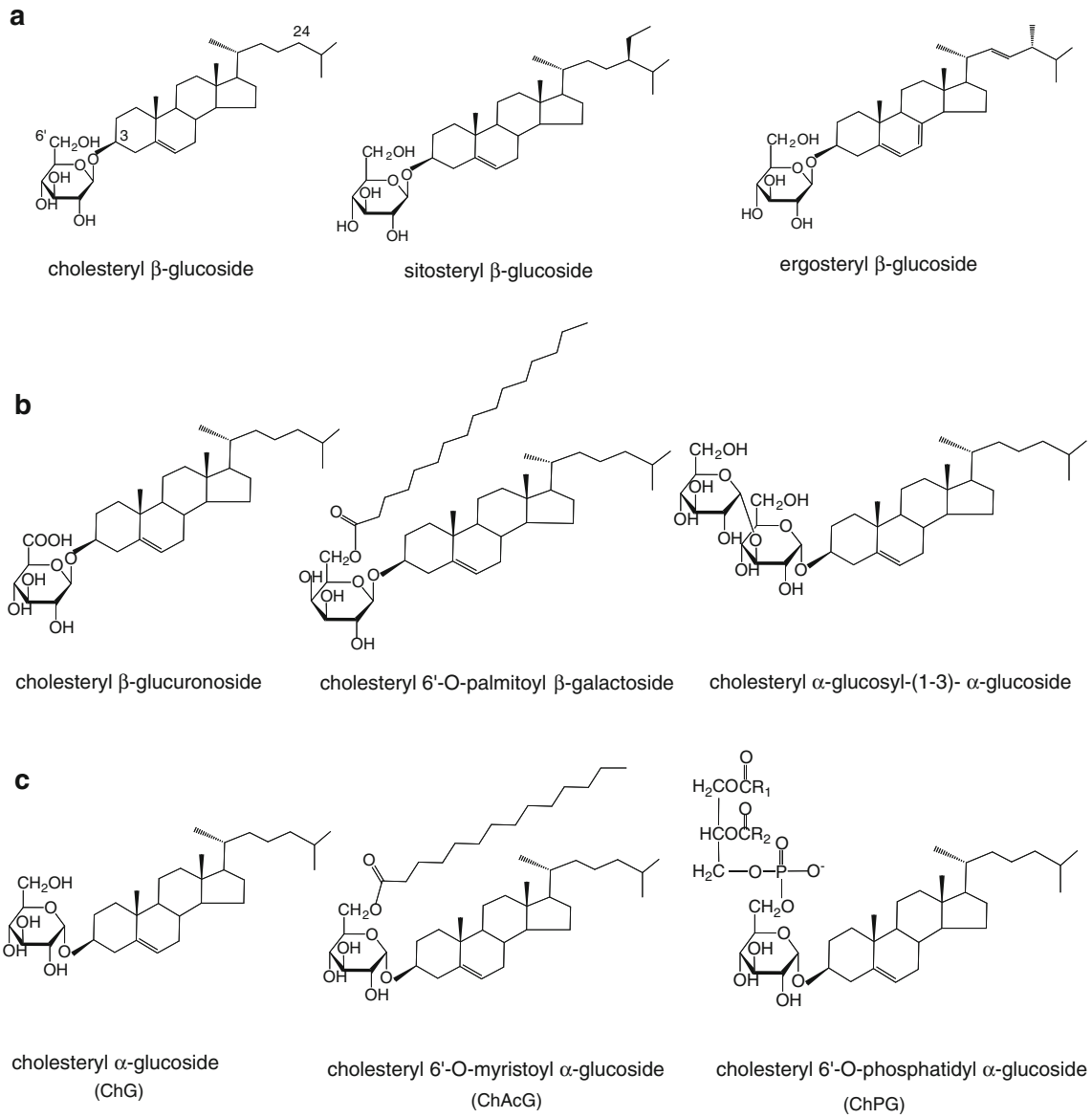

Fig. 1 Structure of typical steryl glycosides. **a** Polymorphism of the sterol moiety in steryl glycosides. Cholesteryl β-glucoside found from a human fibroblast cell line, sitosteryl β-glucoside from plant, and fungal ergosteryl β-glucoside (*Pichia pastoris*) are shown. **b** Polymorphism of glycosides in steryl glycosides. Cholesteryl β-glucuronoside found from the human liver, cholesteryl 6'-*O*-acyl β-galactoside from *Borrelia burgdorferi,* and cholesteryl diglucoside from *Acholeplasma axanthum* are shown. **c** Three steryl glycosides found from *Helicobacter pylori* are shown

except for 3α-hydroxy steryl glycosides isolated from *Mimusops elengi* (Jahan et al. 1995).

Variations in the Sugar Moiety

The sugar species most commonly found in SGs is D-glucopyranose that is attached to the 3-hydroxy group of the sterol in a β-anomeric configuration. Besides β-glucosides, SGs with other sugar species in both α- and β-anomeric configurations also occur in lower abundance (Grill et al. 2010), for instance, β-D-galactopyranose (*Borrelia burgdorferi*) (Stübs et al. 2009), β-D-glucopyranuronic acid (*Homo sapiens*) (Hara and Taketomi 1982; Muhiudeen et al. 1984; Taketomi et al. 1982), β-D-xylopyranose (Sea cucumber) (Elyakov GB et al. 1980), and α-D-glucopyranose (*Helicobacter pylori*) (Hirai et al. 1995). Some SGs consist of a diglycosyl or oligoglycosyl sugar moiety (Kovganko and Kashkan 1999; Grill et al. 2010), for instance, α-Glc(1-3)α-Glc (*Acholeplasma axanthum*) (Mayberry and Smith 1983).

Importantly, acylation is frequently observed at the C6′-primary hydroxy group of the pyranoses constructing SGs with palmitic acid, oleic acid, or other fatty acids, for example, 6′-*O*-palmitoyl β-glucose in soybeans, potatoes (Lepage 1964), and snakes (Abraham et al. 1987). Acylation of SGs not only affects the biophysical properties of cell membranes, such as hydrophobicity, but also the immunogenicity of cells, as will be described in the sections below.

Sterol Glycosyltransferase

The key enzyme responsible for the biosynthesis of SGs is sterol glycosyltransferase which transfers sugars from sugar nucleotides to the 3-hydroxy group of sterols. Cloning of the genes encoding sterol glycosyltransferase has been accomplished from plants, fungi, moulds, and bacteria (Grill et al. 2010), but not from animal cells. Recently, cholesterol glucosyltransferase activity was detected in a heat-shocked human cell line (Akiyama et al. 2011). Interestingly, it has been suggested that this enzyme catalyzes the transfer of a glucose from glucosylceramide, but not from sugar nucleotides, to cholesterol. Gene cloning and further characterization of this enzyme is expected.

Immunological Functions of Steryl Glycosides

Th1 Shift Following Phytosteryl β-Glucoside Administration

The immunological functions of SGs were first suggested from findings that immune responses were affected by the administration of β-steryl glycosides isolated from plants.

When murine helper T cells were stimulated in vitro with phytohemagglutinin, cell proliferation and production of Th1 cytokines, such as IFN-γ and IL-2, were increased in the presence of sitosterolin (a mixture of sitosterol and sitosteryl β-glucoside) (Bouic et al. 1996). Since the amount of sitosterol supplied to the cell culture contributed to the increase in the concentration of sitosterol much less than the physiological serum concentration in healthy subjects, it is likely that the sitosteryl β-glucoside included in sitosterolin was responsible for the induction of Th1-skewed immune responses.

The effects of β-sitosteryl glucoside in vivo were also tested. Mice injected with sitosteryl β-glucoside survived longer than mice untreated following infection with *Candida albicans* (Lee et al. 2007). Since the protective effects were dependent on the presence of $CD4^+$ T cells, depletion of these cells from mice abrogated the effect of SG treatment to prolong the survival of the subjects. Moreover, when spleen lymphocytes prepared from mice previously treated with sitosteryl β-glucoside were stimulated with an anti-CD3 monoclonal antibody ex vivo, they produced five times more IFN-γ and four times more IL-2 than splenocytes from mice without treatment with sitosteryl β-glucoside. These findings suggest that enhanced Th1 immunity in mice treated with sitosteryl β-glucoside is attributable to resistance against *Candida albicans* infection.

The effects of sitosteryl β-glucoside treatment were also observed in humans. Daily administration of sitosterolin accompanied by regular treatment promoted general recovery from pulmonary tuberculosis with increased proliferation of lymphocytes (Donald et al. 1997), while administration of sitosterolin brought beneficial effects to patients suffering from allergic diseases such as rhinitis and sinusitis (Bouic 2001).

Collectively, these results suggest that sitosteryl β-glucoside is a potent immune regulator that can shift the Th1/Th2 balance towards Th1 dominant immunity. Further examinations are required to explore a possible explanation for the mechanism underlying these findings.

Cholesteryl 6′-*O*-Acyl β-Galactoside Produced by *Borrelia burgdorferi* as an Immunogen in Patients with Lyme Disease

The etiological agent of Lyme disease, spirochete *Borrelia burgdorferi,* is usually transmitted to humans by ticks of the genus *Ixodes*. Two glycolipid components of *Borrelia burgdorferi* have been identified that react with sera from Lyme disease patients, α-galactosyl diacylglycerol (Hossain et al. 2001) and cholesteryl 6′-*O*-acyl β-galactoside (Ben-Menachem et al. 2003; Schröder et al. 2003). The minimum essential requirement for the latter component to

be recognized as an immunogenic motif by the antibodies of patients was demonstrated to be galactose, cholesterol, and fatty acids with a minimal chain length of four carbon atoms (Stübs et al. 2009). Cholesteryl 6′-*O*-acyl *β*-galactoside is ubiquitously expressed by *Borrelia burgdorferi* regardless of its genospecies, thus this molecule may be used as a target of vaccination (Stübs et al. 2011). The precise mechanism by which these bacterial glycolipids induce antibody production from hosts have not yet been elucidated.

Glycosylation of Cholesterol Facilitates *Helicobacter pylori* to Evade Immune Surveillance of the Host

Helicobacter pylori is a Gram-negative bacterial pathogen that has colonized the stomach of humans since their early evolution (Linz et al. 2007). Following the first demonstration of the pathogenic potential of *Helicobacter pylori* (Marshall and Warren 1984), accumulating evidence has shown that this bacterium causes gastric ulcers, carcinoma, and mucosal associated lymphoid tissue lymphoma (Ernst and Gold 2000; Uemura et al. 2001; Peek and Blaser 2002). *Helicobacter pylori* is auxotrophic for cholesterol, one of the important components of the cell membrane (Simons and Vaz 2004). *Helicobacter pylori* follows a cholesterol gradient and extracts this lipid from plasma membranes of epithelial cells of the host stomach (Wunder et al. 2006). Incorporation of cholesterol promotes phagocytosis of *Helicobacter pylori* by antigen-presenting cells and enhances the following antigen-specific immune response of the host (Wunder et al. 2006), where Th1 $CD4^+$ T cells play crucial roles (Eaton et al. 1999; Pappo et al. 1999; Lundgren et al. 2005; Nagai et al. 2007).

Helicobacter pylori converts cholesterol to cholesteryl 3-α-D-glucoside (ChG) to evade immune surveillance just a few hours after incorporating cholesterol (Wunder et al. 2006). Most ChG is further converted to cholesteryl 6′-*O*-acyl (mainly $C_{14:0}$ myristyl), α-glucoside (ChAcG), or cholesteryl 6′-*O*-phsphatidyl α-glucoside (ChPG) (Hirai et al. 1995; Wunder et al. 2006), although there have been no reports on the gene cloning of the *O*-acyltransferases for ChG. When *Helicobacter pylori* colonizes gastric mucosa, the bacterial carbohydrate binding protein, adhesin, binds to the host blood type-determining sugar chains (Ilver et al. 1998; Gerhard et al. 1999; Mahdavi et al. 2002). Gastric gland mucus-associated *O*-glycans carrying a terminal GlcNAcα1-4 unit have been demonstrated to inhibit the activity of bacterial cholesterol α-glucosyltransferase (encoded in the *hp0421* gene) and prevent the conversion of cholesterol to its glucosides during the interaction between the bacterium and the host, resulting in the promotion of phagocytosis of invading *Helicobacter pylori* (Kawakubo et al. 2004).

Although conversion of incorporated cholesterol to its glycosides obviously facilitates *Helicobacter pylori* to escape from the immune surveillance of the host as described above, it remains unclear whether cholesteryl glycosides thus produced are directly involved inside this interaction between the host and the invading *Helicobacter pylori* or if ChAcG works in antigen-presenting cells following phagocytosis.

Invariant TCR-Bearing NKT Cell-Targeted Activation of the Immune System with Cholesteryl 6′-*O*-acyl α-Glucoside Produced by *Helicobacter pylori*

It has been suggested that conversion of cholesterol to its glycosides affects the interaction between *Helicobacter pylori* and the host in another way. We have recently demonstrated that ChAcG produced by *Helicobacter pylori* is presented by the non-classical MHC class Ib molecules CD1d following phagocytosis and processing of the microbes by antigen-presenting cells and recognized by invariant TCR-bearing NKT cells.

Murine invariant Vα14-Jα18 TCR^+ and their human counterpart invariant Vα24-Jα18 TCR^+ NKT (*i*NKT) cells are a subset of T lymphocytes (Bendelac et al. 1997) that specifically respond to certain glycolipids in the context of CD1d such as α-galactosyl ceramide (α-GalCer) (Kawano et al. 1997) isolated from marine sponge (Natori et al. 1993), α-glucuronosyl and α-galacturonosyl ceramides from α-proteobacteria (Kinjo et al. 2005; Mattner et al. 2005), and α-galactosyl diacylglycerol from *Borrelia burgdorferi* (Kinjo et al. 2006). In addition to direct stimulation with CD1d-associated exogenous antigens, it has been recently demonstrated that *i*NKT cells are activated with concomitant stimulations with self-antigens such as isoglobotriaosyl ceramide (iGb3) or *β*-GlcCer and IL-12 produced by antigen-presenting cells (Brigl et al. 2011; Brennan et al. 2011). We focused our attention on the structural similarities between ChAcG and those stimulants for *i*NKT cells and tested whether ChAcG was a potent antigen for this particular T cell subset.

We have demonstrated that glycolipid extracts from *Helicobacter pylori* induced immune responses from C57BL/6 liver lymphocytes. The immune responses were CD1-dependent because they were reduced in the presence of anti-CD1 blocking antibody or when liver lymphocytes were prepared from $CD1^{-/-}$ mice. Lipids isolated from the *Helicobacter pylori* mutant line deficient in the *hp0421* gene induced lower immune responses from liver lymphocytes (Shimamura et al. manuscript in preparation). Therefore, cholesteryl α-glucosides were responsible for the induction of immune responses from C57BL/6 liver cells. In fact, ChAcG isolated from *Helicobacter pylori,* or the chemically synthesized form, induced activation of

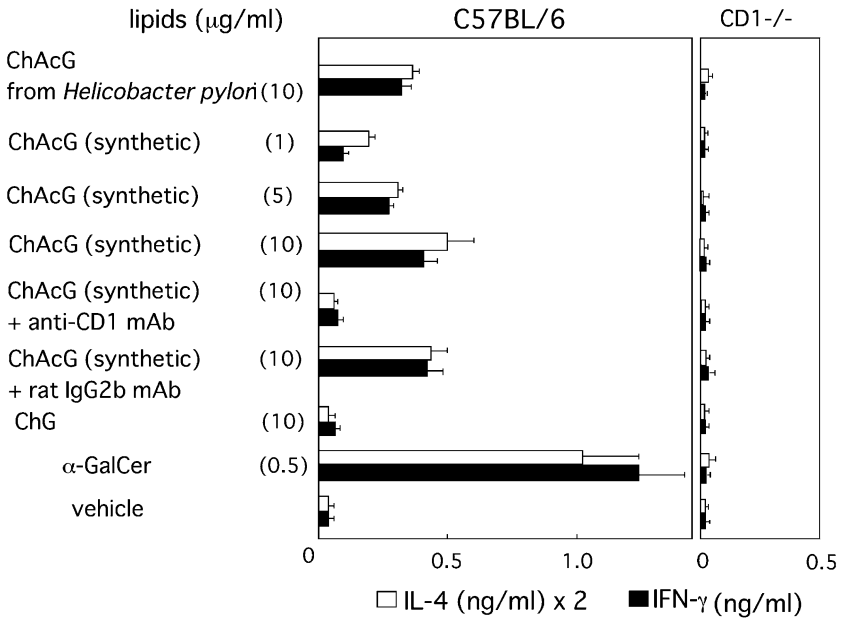

Fig. 2 Cholesteryl acyl glucoside (ChAcG) stimulated subsets of lymphocytes in a CD1-dependent manner. Liver mononuclear cells prepared from C57BL/6 and $CD1^{-/-}$ mice were cultured in the presence of ChAcG isolated from *Helicobacter pylori* of SS1 (a strain contagious to mice), or synthetic ChAcG. Immune responses were assessed by measuring cytokine production by responder cells. Anti-CD1 mAb or isotype-matched rat IgG2b (10 μg/ml) was added to the culture to evaluate the CD1-dependency of immune responses. Mean values of the triplicate cultures are shown

liver lymphocytes in a CD1-dependent manner (Fig. 2). As described in the previous section, *Helicobacter pylori* produces three types of cholesteryl glucoside, ChG, ChAcG, and ChPG (Hirai et al. 1995). Among these *Helicobacter pylori* products, only ChAcG had the potential to activate *i*NKT cells. The lymphocytes responding to ChAcG in the liver were identified to be *i*NKT cells. Hybridomas derived from *i*NKT cells were activated by stimulation with ChAcG that was presented by CD1/*β*2-microglobulin proteins immobilized on culture plates, whereas hybridomas derived from irrelevant $V\beta8^+$ T cells were not (Chang et al. 2011). Similarly, *i*NKT cell hybridomas were activated with ChAcG presented by $CD1d^+$ bone marrow-derived dendritic cells (Fig. 3). ChAcG induces immune responses not only from murine, but also from human invariant TCR α (Vα24-Jα18)-bearing NKT cells in a CD1d-dependent manner (Chang et al. 2011). Collectively, these findings indicate that ChAcG in the context of CD1d is recognized by the semi-invariant TCR and induces activation of *i*NKT cells. To our knowledge this is the first demonstration of recognition of cholesterol derivatives by TCR.

It has been demonstrated that *Helicobacter pylori* spontaneously shapes different forms of colonies, a large (L) form and a small (S) form, depending on the surrounding conditions (Bukholm et al. 1997). These two types of colonies are reproducible in cultures by adjusting the pH of the medium. The relationship between the colony form and the lipid composition of *Helicobacter pylori* has been examined (Tannaes et al. 2000, 2005) (summarized in Fig. 4). *Helicobacter pylori* tends to form S-form colonies under acidic conditions. The up-regulated expression of phospholipase A2 (PLA2) in microbes in the S-form

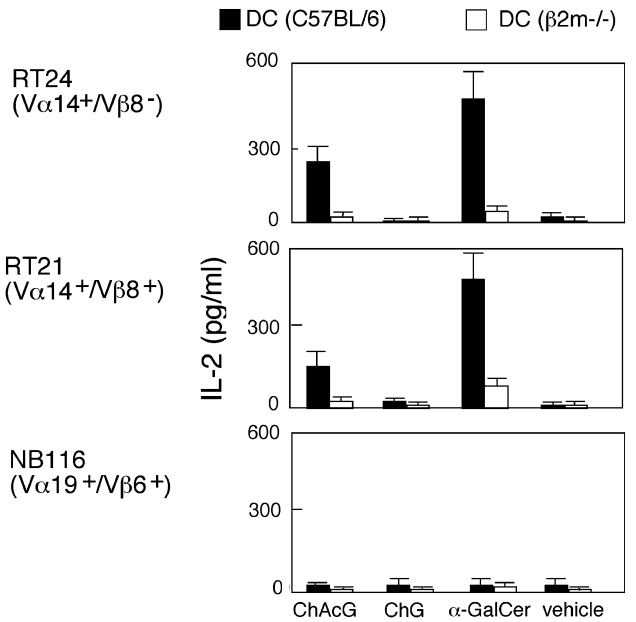

Fig. 3 ChAcG presented by dendritic cells activated invariant NKT cell hybridomas in a CD1-dependent manner. Dendritic cells were prepared from bone marrow cells of C57BL/6 and *β*2-microglobulin-deficient mice (Shimamura et al. 2007). Hybridomas derived from either invariant Vα14 cells (RT24, RT21) (Shimamura et al. 1997) or Vα19 NKT cells (NB116) (Shimamura and Huang 2002) were cultured for 2 days with the dendritic cells. IL-2 in the culture supernatants was determined by ELISA. Mean values of the triplicate cultures are shown

colony induces lyso-phospholipid formation and destabilization of the cell membrane, resulting in the increased release of VacA toxin and urease, an enzyme that is required to survive under strong acidic conditions. Therefore, *Helicobacter pylori* in the S-form colony is likely to

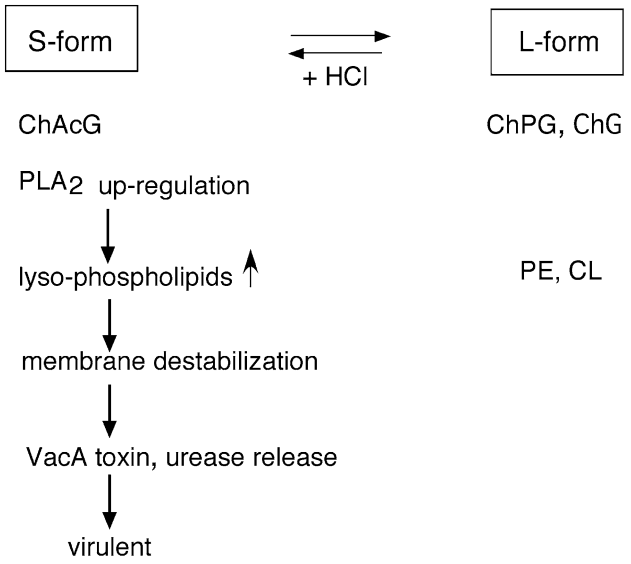

Fig. 4 *Helicobacter pylori* colony variants (S-form and L-form) and lipid composition *PE* phosphatidylethanolamine, *CL* cardiolipin

be more virulent than the bacterium in the L-form colony at the surface of the host stomach. *Helicobacter pylori* in the S-form produces more ChAcG than ChPG or ChG using incorporated cholesterol. In accordance with this cholesteryl glucoside composition, lipids from microbes in the S-form colony induced stronger immune responses from *i*NKT cells than those from the L-form colony (Shimamura et al. manuscript in preparation), suggesting that immune surveillance by *i*NKT cells is of importance toward *Helicobacter pylori* in a virulent state.

Taken together, these findings imply that *Helicobacter pylori* that has converted cholesterol to its glycosides to get rid of phagocytosis is still under the surveillance of the host immune system, where recognition of *Helicobacter pylori* ChAcG by *i*NKT cells triggers the following immune responses. From this point of view, a recent report on the accumulation of $CD161^+$ NKT-like cells at the sites of infection by *Helicobacter pylori* in the stomach is suggestive (O'Keeffe et al. 2008). Currently, a project to prove this possibility is in progress by our group.

It was recently reported that cholesteryl glycosides were localized in the outer rather than the inner membrane of *Helicobacter pylori* (Shimomura et al. 2009). This observation was supported by the following report where activation of cholesterol α-glucosyltransferase in the outer membrane of *Helicobacter pylori* was demonstrated (Hoshino et al. 2011). It is likely that localization of ChAcG in the outer membrane of *Helicobacter pylori* allows the host to recognize *Helicobacter pylori* directly as a target using any antigen receptors for pathogen-associated molecular patterns expressed on the surface of microbes. In fact, we have recently found that ChAcG induces immune responses (production of TNF-α and IFN-γ, but not IL-4) even from liver mononuclear cells of β2 microglobulin-deficient mine (which lack generation of *i*NKT cells), and that one of the innate antigen receptors expressed by antigen-presenting cells is involved in the recognition of ChAcG (Shimamura and Yamasaki, manuscript in preparation).

Immunoregulation by ChAcG Administration via Activation of iNKT Cells

We have demonstrated that administration of ChAcG to neonatal mice induces activation of a subset of *i*NKT cells with a phenotype of CD4, CD8-double negative that are characterized by the production of Th1-biased cytokines, and that this induction results in a reduction in the incidence of allergic asthma after maturation (Chang et al. 2011; summarized in Fig. 5). We have also found that production of anti-OVA antibodies of Th2-controlled isotypes such as IgE and IgG1, but not Th1-controlled IgG2a,

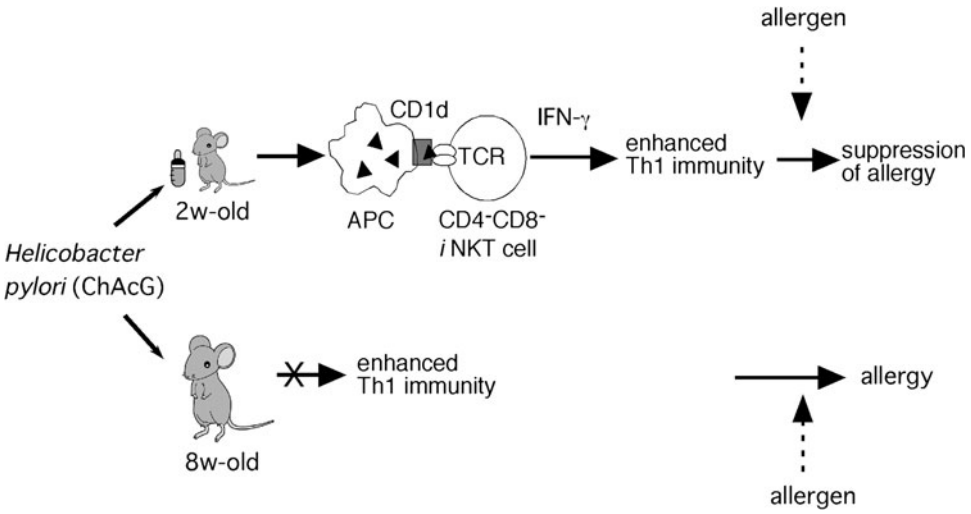

Fig. 5 Administration of ChAcG to newborns protects them from the onset of allergies after maturation. Injection of ChAcG to neonate (2 weeks old), but not adult (8 weeks old) mice reduces the incidence of OVA-induced allergic asthma. Details are available in the original report (Chang et al. 2011)

is suppressed in mice immunized with OVA when they were subjected to administration of ChAcG prior to immunization (Shimamura et al. unpublished results). These observations strongly suggest that injection of ChAcG induces activation of Th1 immunity from the host and contributes to homeostasis of the immune system to protect the host from allergy or pathogenic infection.

Concluding Remarks

The immunoregulatory functions of SGs are summarized in this review such as the induction of Th1 immunity by sitosteryl β-glucoside, the rise in antibody titer against cholesteryl β-galactoside following *Borrelia burgdorferi* infection, and evasion of *Helicobacter pylori* from immune surveillance by converting extracted cholesterol to α-glucosides. Each observation is very interesting. However, it is still unclear how these SGs are recognized by the host immune system and where they exert their function, on the surface of the host cells or inside the cells after incorporation. On the other hand, mechanisms for the recognition of *Helicobacter pylori* ChAcG by the host immune system have been very partially revealed; ChAcG is presented by CD1d and recognized by *i*NKT cells. Further investigations are obviously required for us to determine the contribution of this recognition mechanism to protection against *Helicobacter pylori*.

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Conflict of interest The author declares that he has no conflict of interest.

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