Scavenger receptors target glycolipids for natural killer T cell activation

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NKT cells are innate-like T cells with powerful regulatory functions that are a promising target for immunotherapy. The efficacy of glycolipids, such as the prototypic NKT cell antagonist α-galactosylceramide (αGalCer), is currently being evaluated in clinical trials, but little is known about factors that target lipid antigens for CD1d presentation and NKT cell activation in vivo. Lipid uptake via the LDL receptor (LDLR) has been shown for digalactosylceramide; however, whether this pathway contributes to CD1d presentation of other important NKT cell agonists remains unclear. We therefore investigated receptor-mediated uptake pathways for CD1d presentation using a panel of structurally diverse lipid antigens. We found that uptake via scavenger receptors was essential for the CD1d presentation of αGalCer and *Sphingomonas* glycolipids. Moreover, in vivo NKT cell responses, i.e., cytokine production, proliferation, and NKT cell help for adaptive CD4+ and CD8+ T cells, required the uptake of αGalCer via scavenger receptor A. Importantly, our data indicate that structural characteristics of glycolipids determine their receptor binding and direct individual lipids toward different uptake pathways. These results reveal an important contribution of scavenger receptors in the selection of lipids for CD1d presentation and identify structural motifs that may prove useful for therapeutic NKT cell vaccination.

Introduction

The efficacy of current vaccines depends on adjuvants, microbial products that activate the innate immune system by triggering pattern recognition receptors (PRRs), such as TLRs or NOD-like receptors (NLRs) (1–4). The resulting inflammatory response and maturation of professional APCs is essential for the priming of adaptive T cell immunity (3, 5). NKT cells represent a specialized subset of innate-like T lymphocytes that recognize glycolipid antigens presented by CD1d (6–8). NKT cells have gained particular interest because of their potent immunoregulatory functions. Upon activation, NKT cells rapidly secrete a diverse array of cytokines and induce the maturation of professional APCs (9, 10). Hence, NKT cells have an impact on the initial phase of the adaptive immune response and are regarded as a "natural adjuvant." In fact, NKT cell agonists substantially enhance the priming of T and B cell responses (11–13) and confer increased protection against tumors in experimental studies (14, 15). As a consequence, NKT cell activation has emerged as a promising strategy for novel vaccination approaches (10, 16), and the efficacy of lipid antigens, such as the prototypic NKT cell agonist α-galactosylceramide (αGalCer), is currently being evaluated in cancer patients in clinical trials (16, 17). However, little is known about host factors that determine the in vivo bioactivity of NKT cell agonists and target lipids into the CD1d presentation pathway. A pivotal role of the LDL receptor–mediated (LDLR-mediated) uptake has been proposed for the directing of antigens toward CD1d presentation. In particular, it was shown that presentation of the digalactosylceramide antigen αGal(α1-2)GalCer required its binding to VLDL particles and subsequent uptake via the LDLR in an apoE-dependent manner (18). Yet it remains unclear whether this pathway also

controls the CD1d presentation of other lipid antigens, such as the clinically relevant NKT cell agonist αGalCer.

Scavenger receptors (SRs) comprise a large family of structurally diverse molecules that function as PRRs for both modified self and pathogenic non-self molecules (19). While first members of this family, such as SRA and CD36, were initially characterized as macrophage receptors for modified lipoproteins (20–22), the important contribution of SRs to innate immune responses is now widely recognized (19, 23, 24). For example, SRs mediate the phagocytic uptake of Gram-negative and Gram-positive bacteria, viruses, and parasites (24). In addition, SRs recognize isolated microbial components, such as LPS (25), lipoteichoic acid (26), or CpG DNA (27), and collaborate with TLRs 2, 4, and 6 in the recognition of lipid TLR agonists (28, 29). Accordingly, SR-deficient mice exhibit increased susceptibility to microbial infection (28, 30–32). Furthermore, the SR-mediated uptake of antigens has been linked to MHC presentation, suggesting that SRs may also influence the priming of adaptive immune responses (33, 34). Thus, SRs bind a wide range of ligands associated with microbial infection and inflammatory disease, cooperate with TLRs to diversify the specificity of pathogen pattern recognition, and couple to MHC antigen presentation for the induction of adaptive T cell immunity.

Here, we have investigated receptor-mediated uptake pathways for CD1d presentation using a panel of structurally distinct lipid antigens and found that only a subset of these agonists depended on uptake via the LDLR. Instead, we show that the SRs SRA, SRB1, and CD36 target lipids toward CD1d antigen presentation. In particular, the CD1d presentation of clinically relevant NKT cell agonists, such as αGalCer and bacterial glycosphingolipids, required antigen uptake via the SRA, but not the LDLR. These results define structural characteristics that direct lipids toward selective uptake via the SR versus LDLR pathways, which could be used to deliberately target antigens for CD1 presentation. Thus, our findings
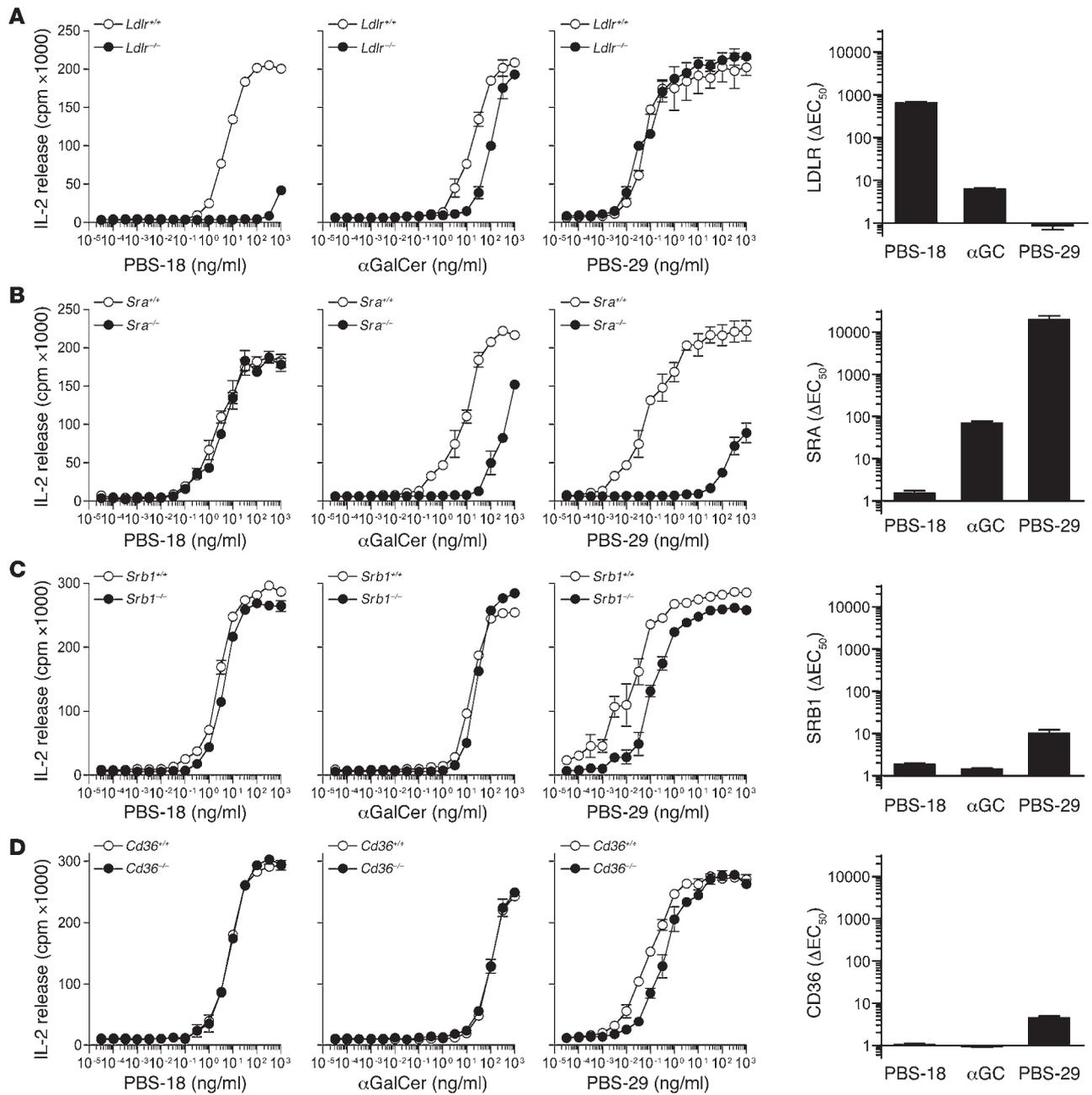
Figure 1
SRs are required for CD1d presentation of αGalCer and of bacterial glycolipid antigen. (**A–D**) CD1d presentation of the indicated NKT cell agonists to the Vα14+ NKT cell hybridoma DN32.D3 by bone marrow–derived DCs in vitro. DCs were derived from receptor-deficient mice (black circles) lacking expression of either (**A**) *Ldlr*, (**B**) *Sra*, (**C**) *Srb1*, or (**D**) *Cd36* or from the respective WT control mice (white circles, **A–D**). The dose-response curves for each lipid antigen were used to calculate the ΔEC_{50} for each of the uptake receptors. PBS-18, αGal(α1-2)GalCer; PBS-29, synthetic *Sphingomonas* αGluACer. Mean ± SEM values of triplicate determinations representative for 1 of at least 3 independent experiments are shown.

illustrate the complexity of antigen selection for CD1d presentation and link an important class of pathogen recognition receptors to the acquisition of lipids for CD1d antigen presentation.

Results

CD1d presentation of αGalCer and Sphingomonas lipids requires antigen uptake via SRs. To study the receptor-mediated uptake of lipid antigens for CD1d presentation, we examined the ability of professional APCs to stimulate the Vα14+ NKT cell TCR in vitro. For this purpose, DCs from receptor-deficient and WT mice were pulsed with titrated amounts of lipid antigen, washed, and then used to stimulate DN32.D3 NKT hybridoma cells (Figures 1 and 2). As expected (18), the expression of the LDLR was critical for CD1d presentation of the digalactosylceramide αGal(α1-2)GalCer (PBS-18) (35), and the effective concentration (EC_{50}) for PBS-18 increased 647-fold in the absence of the LDLR (Figure 1A). Even
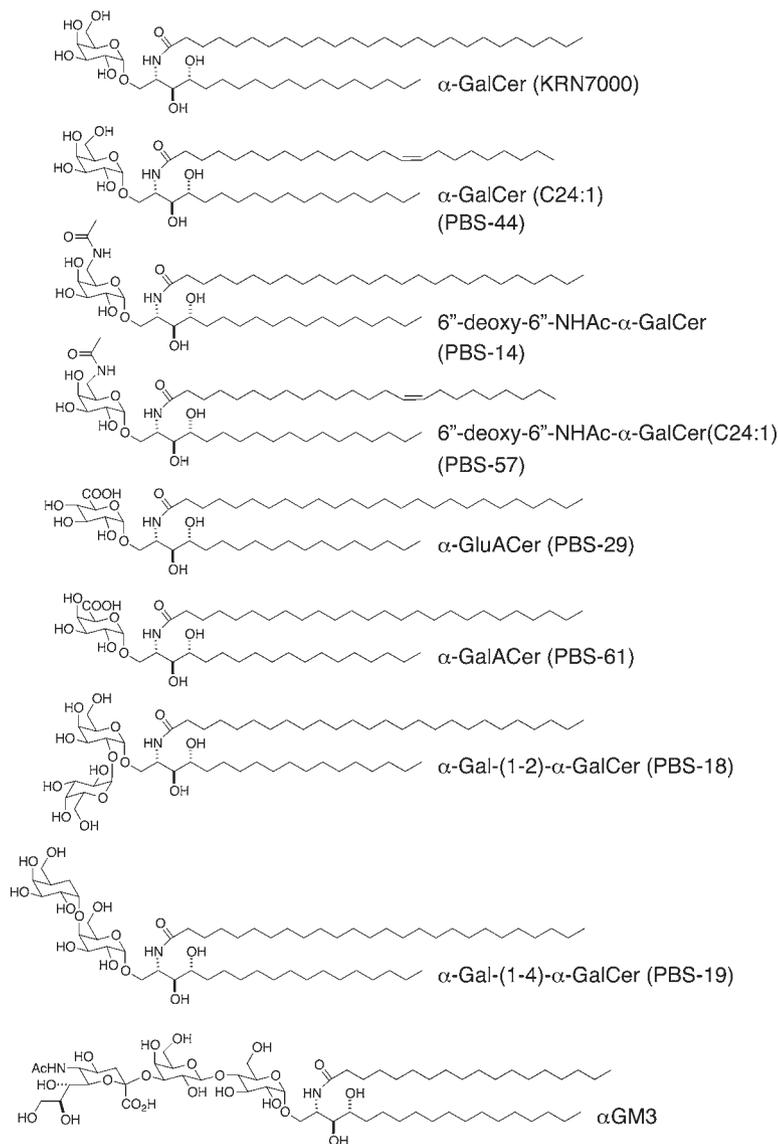
Figure 2
Structures of the NKT cell agonists used in this study.

a ΔEC_{50} of 69.2 versus 6.2 for SRA-mediated versus LDLR-mediated uptake, respectively, whereas LDLR deficiency only slightly influenced the CD1d presentation of αGalCer (Figure 1, A and B). Moreover, the CD1d presentation of bacterial GSL-1 (PBS-29) completely depended on the SR pathway (Figure 1, B–D). $Sra^{-/-}$ DCs were 4 orders of magnitude less efficient in presenting PBS-29 via CD1d than WT cells (ΔEC_{50} = 19,770; Figure 1B). In addition, a reduced CD1d presentation of PBS-29 — albeit to a lesser degree — was also observed in DCs deficient for the class B SRs SRBI (Figure 1C) or CD36 (Figure 1D), whereas neither of these receptors influenced the CD1d presentation of PBS-18 or αGalCer. Thus, analogous to the role of LDLR-mediated uptake for CD1d presentation of the digalactosylceramide antigen (PBS-18), SRs were essential for the CD1d presentation of the prototypic NKT cell agonist αGalCer and of bacterial *Sphingomonas* GSL-1 (PBS-29). Taken together, these findings demonstrate that in professional APCs, multiple receptor pathways contribute to the cellular acquisition of lipid antigens for CD1d presentation.

The chemical structure of the lipid antigen head group defines the receptor uptake pathway. Our data indicated that the LDLR and SR pathways differentially contributed to the cellular uptake of glycolipid antigens for presentation to NKT cells. We next assessed the receptor specificity of a panel of additional NKT cell agonists (Figure 2) in order to define structural determinants that target lipid antigens for cellular uptake via the LDLR or the SRA (Figure 3). As observed for the digalactosylceramide PBS-18 (Figure 1), the digalactosylceramide αGal(α1–4)GalCer (PBS-19) also required uptake via the LDLR, but was presented normally by $Sra^{-/-}$ DCs (Figure 3). Thus, while presentation of αGalCer appeared to involve both uptake via the LDLR and the SRA, the addition of a second galactose moiety strongly increased the LDLR-mediated uptake of the resulting lipid variant and abolished its uptake via the SRA. Similarly, the balance between LDLR and SRA uptake of αGalCer could be easily perturbed by modifying the antigen head group and/or acyl chain. The potent NKT cell agonist PBS-57 (38) was evolved from αGalCer by the combination of 2 modifications: the addition of an acetamide group at the C6″ position of the galactose (PBS-14) and the introduction of a single unsaturation on the acyl chain (PBS-44). Both modifications made PBS-14, PBS-44, and PBS-57 much more LDLR dependent than αGalCer (Figure 3, A and B). However, only PBS-44, which has the same head group as αGalCer, remained SRA dependent, whereas PBS-14 and PBS-57 became impervious to the removal of SRA from the presenting cells (Figure 3). Conversely, the bacterial αGluACer compound and the structurally related *Sphingomonas* αGalACer (PBS-61) depended almost exclusively on uptake via the SRA but not the LDLR. This result could have been anticipated based on the known preference of SRs for negatively charged molecules (24) and given that both SRA-dependent bacterial glycuronylceramides displayed a carboxyl group on the head group. We further assessed the influence of simple modifications of the antigen head group, i.e., size and/or

though this confirmed the LDLR-mediated uptake of the digalactosylceramide antigen, $Ldlr^{-/-}$ DCs still efficiently presented the prototypic NKT cell agonist αGalCer, indicating that αGalCer was only in part taken up via the LDLR pathway (Figure 1, A and B). Furthermore, the *Sphingomonas*-derived α-glucuronosylceramide GSL-1 (αGluACer, PBS-29) (36, 37) was presented equally well by $Ldlr^{-/-}$ DCs; hence, LDLR-mediated uptake played no significant role for CD1 presentation of PBS-29 (Figure 1, A and B). Since these results clearly suggested LDLR-independent pathways for the uptake of αGalCer and PBS-29, we next investigated the potential involvement of members of the SR family, which are known to bind to native and modified lipoproteins. In contrast with its strong dependency on LDLR-mediated uptake, the CD1d presentation of PBS-18 did not require expression of any of the SRs tested and appeared to exclusively depend on the LDLR (Figure 1, B–D). In contrast, we noted a substantial defect of SRA-deficient DCs to present αGalCer (Figure 1B), as illustrated by
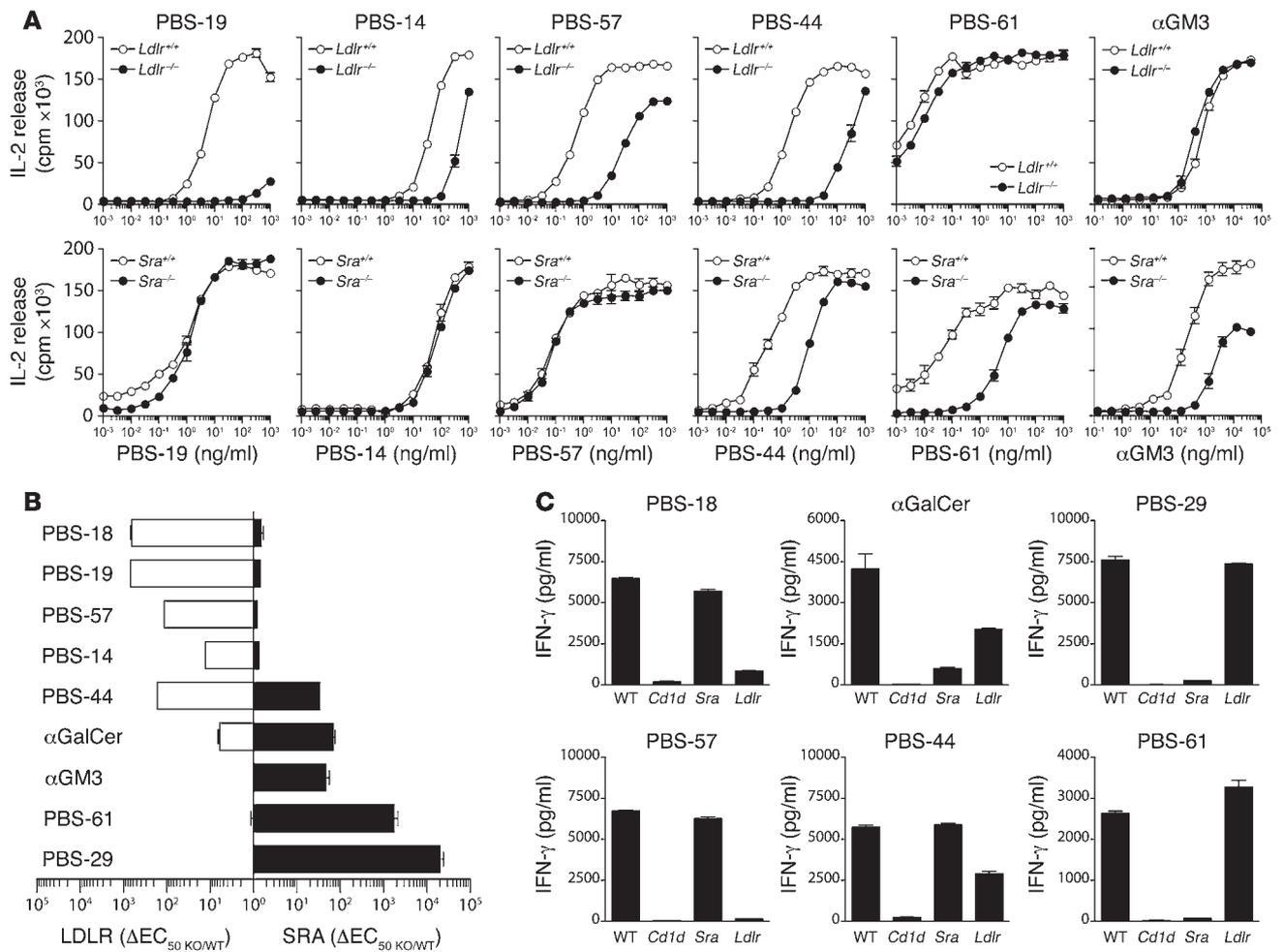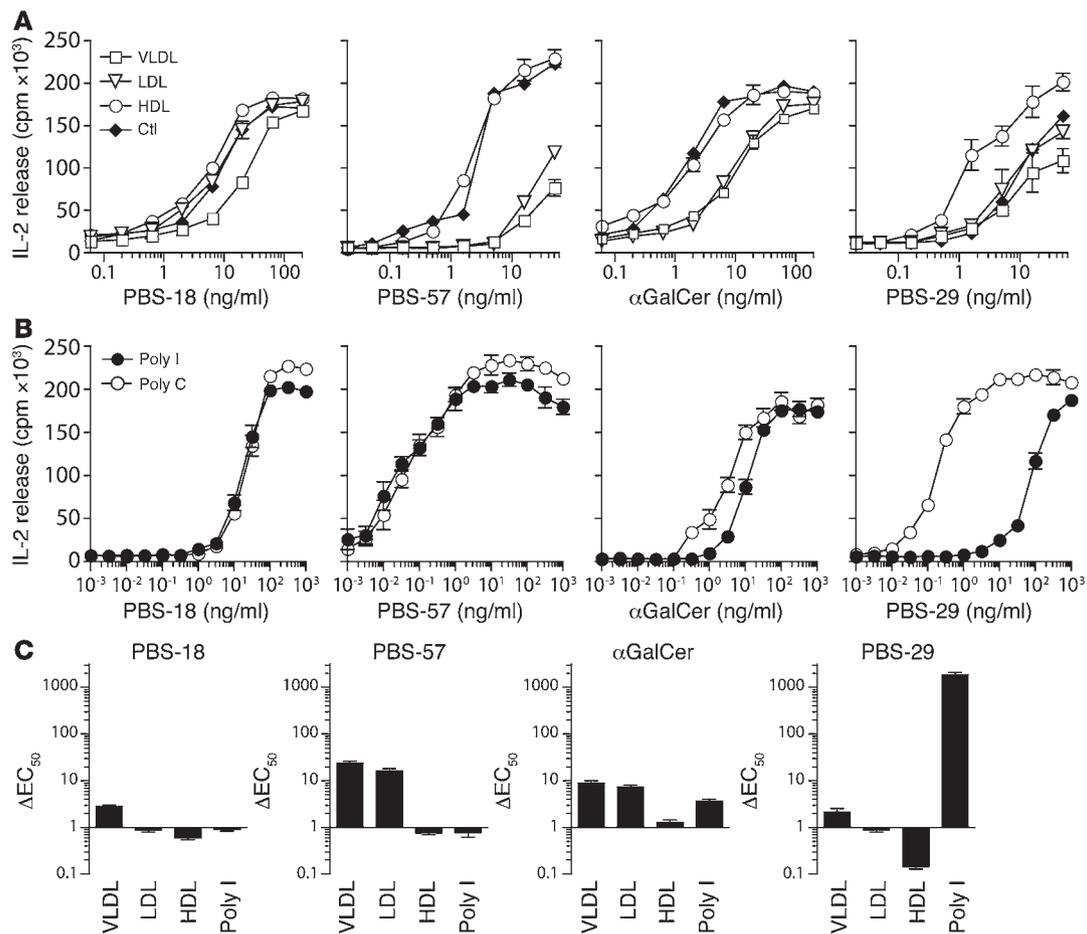
Figure 3
The requirement for SRA or LDLR for CD1d presentation of NKT cell agonists is determined by the chemical structure of the lipid antigen head group. (**A**) CD1d presentation of a panel of NKT cell agonists (chemical structures are depicted in Figure 2) to DN32.D3 NKT hybridoma cells by $Ldlr^{-/-}$ DCs (black circles, top panels) and by $Sra^{-/-}$ DCs (black circles, bottom panels) as compared with the respective WT DCs (white circles, both panels). (**B**) The dose-response curves for the data shown in Figure 1 and part **A** were used to calculate the ΔEC_{50} in order to compare the requirements of individual lipid antigens for uptake via the LDLR or the SRA pathway. (**C**) CD1d presentation of indicated NKT cell agonists by WT, $Cd1d^{-/-}$, $Sra^{-/-}$, and $Ldlr^{-/-}$ DCs to primary WT NKT cells in vitro. Data represent the mean ± SEM of triplicate determinations from 1 of 3 (**A** and **B**) or 2 (**C**) independent experiments.

charge, for receptor uptake by testing αGM3. αGM3 is a GM3-like ganglioside synthesized from α-anomeric glucosylceramide with the addition of the 2 distal GM3 sugars (Figure 2), which stimulates NKT cells after lysosomal processing. Since αGM3 displays a bulky head group, it could be the target of LDLR-mediated uptake; alternatively, it could be proposed that because of the negatively charged sialic acid, the SRA should dominate its cellular uptake. Indeed, the second hypothesis was proven true, since CD1d presentation of αGM3 did not require LDLR expression but was substantially reduced in the absence of the SRA. Together, these results indicated that subtle modifications of αGalCer are sufficient to alter the cell-surface receptor uptake by APCs. Our data suggested that a bulky head group targeted the resulting lipid antigen toward LDLR-mediated uptake, whereas the introduction of a negative charge favored uptake via SRs. We concluded that the chemical structure of the lipid antigen determined the receptor specificity of the uptake.

It appeared from these experiments that lipid antigens could be classified into at least 3 groups according to the receptor requirements of their uptake for CD1d presentation: (a) LDLR-dependent lipids, including PBS-18 and PBS-57; (b) lipids such as αGalCer and PBS-44, which can be taken up via both LDLR and SRA; and (c) lipids such as PBS-29 and PBS-61, which are exclusively SR dependent (Figure 3B). These results were also recapitulated using the activation of primary NKT cells in in vitro cocultures as a readout (Figure 3C). However, the in vitro IFN-γ response of primary NKT cells did not reveal the contribution of SRA to the uptake of PBS-44, whereas SRA-mediated uptake appeared to be more critical for presentation of αGalCer, indicating a differential contribution of both receptors to the uptake of these lipids in vitro.

Since our previous experiments were performed with receptor-deficient DCs in which CD1d presentation might potentially be impaired due to a disturbed lipid metabolism or lysosomal dysfunction (39), we also explored our findings by interfering with
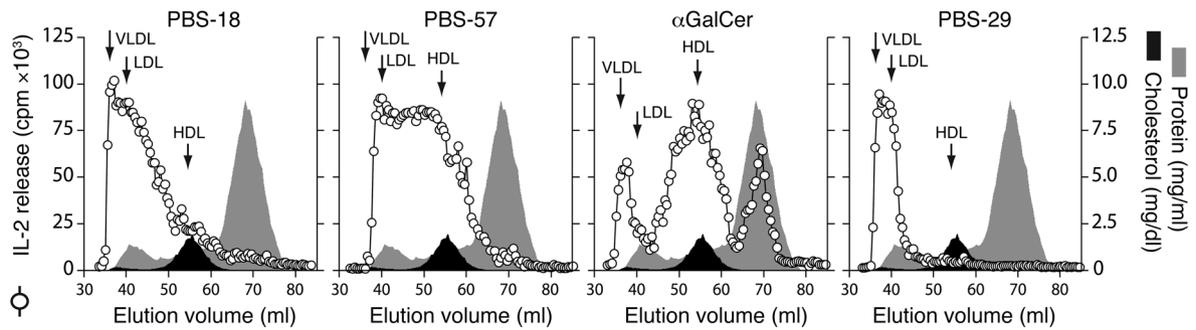
Figure 4
The receptor specificity of lipid antigen uptake is confirmed by blocking lipoprotein receptors and SRs on WT DCs. Presentation of indicated lipid antigens by C57BL/6-derived DCs pretreated (**A**) with murine VLDL, LDL, or HDL to saturate lipoprotein receptors or (**B**) with Poly I to block SR-mediated uptake pathways. (**C**) The ΔEC_{50} of each lipid in the presence of the indicated competitors was calculated from the dose-response curves in **A** and **B**. Pretreatment with polycytidylic (Poly C) was used as a negative control. Mean ± SEM values of triplicate determinations are shown from 1 of 3 independent experiments.

the lipoprotein receptor– or SR-mediated uptake on WT DCs. In order to do so, C57BL/6-derived DCs were pulsed with murine VLDL, LDL, or HDL under serum-free conditions for 30 minutes before titrated amounts of the respective antigens were added and the lipid pulse was continued in the presence of the blocking lipoproteins for an additional 60 minutes. Lipoproteins and lipid antigens were then removed by thorough washes, and CD1d-presented lipid antigens were detected using DN32.D3 NKT cell activation as a reporter system. As anticipated, the CD1 presentation of PBS-18 was inhibited by the addition of VLDL particles, but remained unaffected by competition with LDL or HDL (Figure 4). The uptake of PBS-57 and αGalCer was inhibited by both VLDL and LDL, whereas the presence of HDL had no influence. Contrary to these LDLR-dependent lipids, the CD1d presentation of the exclusively SR-dependent glycolipids such as PBS-29 was not significantly altered by addition of an excess of VLDL or LDL, but a slightly enhanced response to PBS-29 was noted in the presence of HDL (Figure 4A). Thus, saturating lipoprotein receptors on WT DCs with isolated lipoproteins confirmed the data obtained with receptor-deficient cells, which indicated that receptors mediating

the uptake of VLDL and LDL particles contributed to the CD1d presentation of several, but not all, lipid antigens (Figure 4A).

Complementary results were obtained by blocking SR-dependent pathways using polyanionic ligands, such as polyinosinic acid (poly I) (Figure 4B) or fucoidan (not shown). In particular, while presentation of strongly LDLR-dependent lipids, such as PBS-18 and PBS-57, was not affected by pretreatment of DCs with poly I (Figure 4), this markedly reduced the CD1d presentation of αGalCer and drastically impaired the activation of NKT cells by PBS-29. Since these results were obtained by short-term blocking of the receptors on WT cells, they demonstrated that the impaired CD1d presentation of receptor-deficient DCs was not caused by a perturbed lipid homeostasis, but instead resulted from the absence of the respective uptake pathway.

The chemical structure of lipid antigens rather than their lipoprotein association determines the receptor uptake pathway. Our results established that different cell-surface receptors target lipid antigens for CD1d presentation and demonstrated that the specificity of this uptake was influenced by the structural characteristics of the antigen. However, we considered that in vivo, lipid antigens most likely
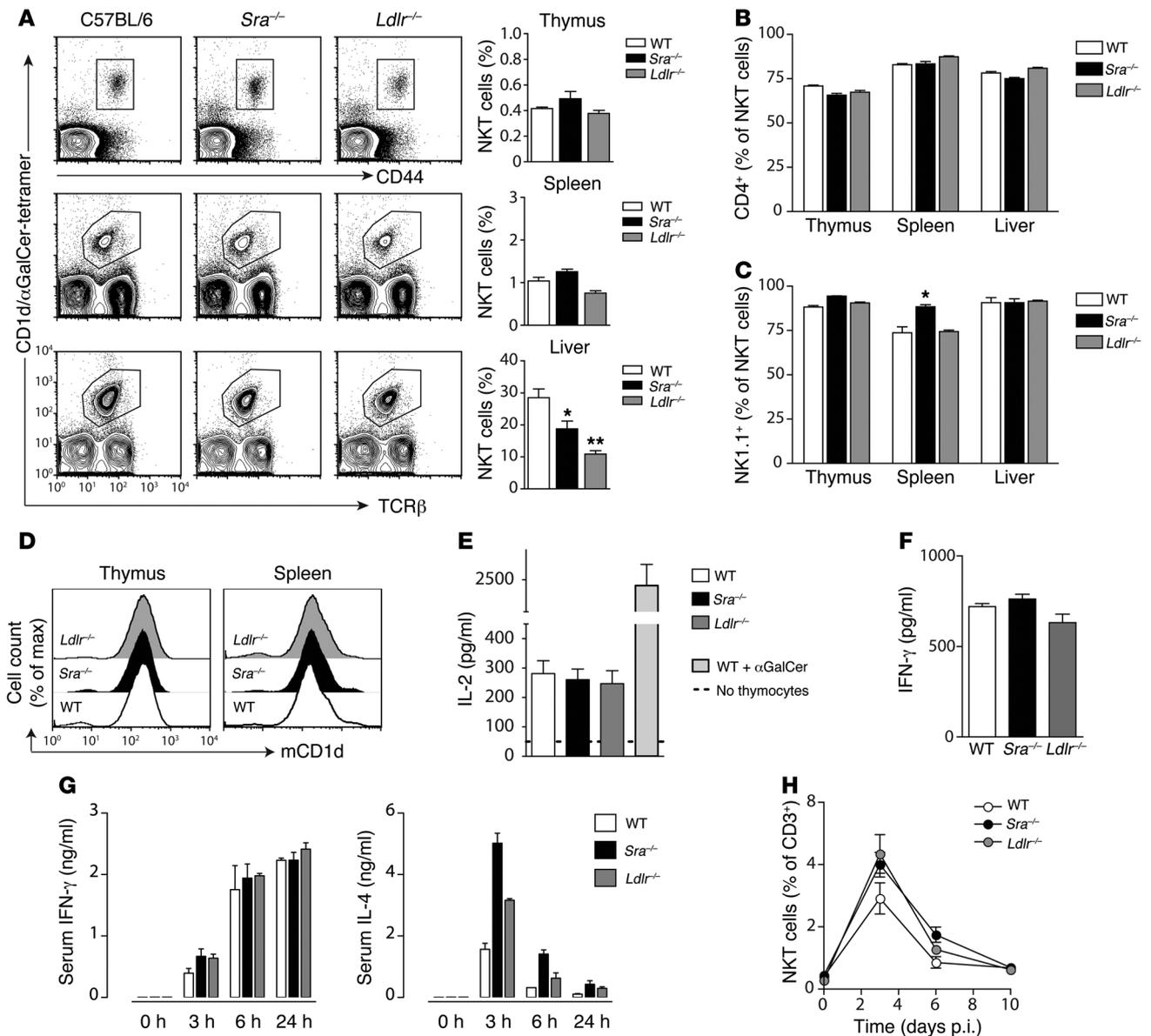
Figure 5
Lipid antigens with different receptor uptake specificities have distinct but overlapping patterns of association with lipoproteins and proteins in serum. Distribution of indicated lipid antigens in murine serum (white circles) after fractionation by gel filtration chromatography as detected by the NKT cell hybridoma DN32.D3 in an antigen presentation assay. The presence of lipoproteins and proteins was determined by the cholesterol (black shaded areas) and protein (gray shaded areas) concentrations of the individual fractions. Data are representative of at least 2 independent experiments.

will be transported bound to lipoprotein particles (18) or serum proteins (40). The differential uptake of structurally distinct lipid antigens by cell-surface receptors could therefore either indicate the direct recognition of the lipid antigen itself (Figure 3) or rather reflect a preferential association of certain lipid structures with particular lipoproteins or serum proteins that in turn would determine the receptor specificity of the uptake. To evaluate both possibilities, we compared the serum distribution of 4 representative lipids that depended on uptake via the LDLR (PBS-18, PBS-57), the SRA (PBS-29), or both receptors (αGalCer) in order to be efficiently presented via CD1d in vitro. Murine serum was pulsed with lipid antigen in vitro for 3 hours, fractionated by gel filtration chromatography, and tested for the presence of the bioactive glycolipids in each fraction using NKT cell activation as a functional readout (Figure 5). Consistent with its LDLR-mediated uptake, PBS-18 co-eluted with the VLDL and LDL fractions (Figure 5). Similarly, PBS-57, which was comparable to PBS-18 in its strong LDLR dependency, preferentially associated with VLDL and LDL fractions (Figure 5). While both strictly LDLR-dependent lipid antigens associated with lipoproteins that would target uptake via the LDLR, αGalCer not only distributed to the VLDL/LDL fractions, but also co-eluted with HDL and the pool of nonparticulate serum proteins (40). However, the exclusively SRA-dependent PBS-29 exhibited an almost identical serum distribution as the LDLR-dependent PBS-18 and was detected only in the VLDL/LDL fractions (Figure 5). Thus, provided that several structurally distinct lipid antigens targeted completely different uptake pathways but co-eluted with the same serum lipoprotein fractions, we concluded that it was not the preferential association with certain lipoproteins that guided the uptake of bioactive glycolipids, but rather the direct recognition of structural features of the antigen by cell-surface receptors.

Functional NKT cell populations in receptor-deficient mice. Collectively, our results had thus far shown that lipid antigens can enter the CD1 pathway via LDLR or SRA and identified SRA as a main receptor involved in the targeting of αGalCer and PBS-29 toward CD1 presentation in vitro. To evaluate the relative contribution of these receptors to other aspects of NKT cell biology, we analyzed NKT cell homeostasis and activation in $Ldlr^{-/-}$ and $Sra^{-/-}$ mice. Absence of LDLR or SRA did not significantly affect the thymic or splenic NKT cell populations (Figure 6A). However, the numbers of liver-resident NKT cells were reduced by 62% and 34% in $Ldlr^{-/-}$ and $Sra^{-/-}$ mice, respectively. While the lack of either recep-

tor did not change the CD4 expression of the different NKT cell subsets, we observed a slightly increased proportion of splenic NK1.1+ NKT cells in $Sra^{-/-}$ mice (Figure 6, B and C). Neither of the 2 receptors influenced the CD1d expression on thymocytes and splenocytes (Figure 6D) or the presentation of endogenous lipid antigens (Figure 6E), and receptor-deficient mice had normal populations of conventional T cells (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI62267DS1). In addition, NKT cells isolated from the receptor-deficient mice produced WT levels of IFN-γ upon stimulation with αGalCer-loaded WT DCs in vitro (Figure 6F). Finally, despite the reduced number of liver-resident NKT cells, both $Sra^{-/-}$ and $Ldlr^{-/-}$ mice responded normally to priming with αGalCer-pulsed WT DCs in vivo, as assessed by the NKT cell–dependent serum IFN-γ and expansion of the NKT cell population, and exhibited rather enhanced IL-4 responses (Figure 6, G and H). Thus, lack of SRA or LDLR expression modulated the size of the liver-resident NKT cell subset; however, it did not compromise the ability of the receptor-deficient NKT cells to mount physiological responses in vivo or in vitro, provided that the lipid antigen was efficiently presented by CD1d on WT APCs.

Dysfunctional NKT cell activation in response to soluble lipid antigen. Having verified the functionality of the NKT cell populations in the deficient mice, we examined their in vivo response to priming with soluble lipid antigen to evaluate the role of the respective receptors for the uptake of glycolipids by professional APCs in vivo. We focused our attention on αGalCer, since we had shown that it could be taken up via both receptors in vitro (Figures 1 and 4). IL-4 production was almost completely abrogated in $Ldlr^{-/-}$ as well as in $Sra^{-/-}$ mice, which indicated that both receptors contributed to the priming of the physiological NKT cell cytokine response. However, $Ldlr^{-/-}$ mice produced WT levels of NKT cell–dependent IFN-γ, though with delayed kinetics, whereas the IFN-γ responses were severely impaired in the absence of SRA expression. Furthermore, NKT cell expansion was normal in LDLR-deficient mice after immunization with αGalCer, but significantly reduced in αGalCer-injected $Sra^{-/-}$ mice (Figure 7). Thus, while both receptors were required for stimulating NKT cells to produce IL-4, the αGalCer-elicited IFN-γ response and NKT cell expansion appeared to selectively require antigen uptake via the SRA but not the LDLR. Considering that the potent adjuvanticity of NKT cell activation represents a major incentive for the therapeutic use of NKT cell agonists in human patients, we next

Figure 6
Functional NKT cell responses in *Ldlr⁻/⁻* and *Sra⁻/⁻* mice. (**A–C**) NKT cell populations in thymus, spleen, and liver of naive *Sra⁻/⁻*, *Ldlr⁻/⁻*, and WT mice were identified by (**A**) CD1d/αGalCer-tetramer staining and further characterized by the surface expression of (**B**) CD4 and (**C**) NK1.1. (**D**) CD1d expression on thymocytes and splenocytes of *Sra⁻/⁻*, *Ldlr⁻/⁻*, and WT mice. FACS plots of a single, representative mouse per strain are shown. Bar graphs depict the mean ± SEM for groups of 4–6 mice from 1 of 3 independent experiments. (**E**) CD1d presentation of endogenous lipid antigens to NKT cell hybridoma cells by thymocytes of indicated strains. (**F–H**) The response of *Ldlr⁻/⁻*, *Sra⁻/⁻*, and WT NKT cells to stimulation with in vitro αGalCer-pulsed WT DCs was assessed (**F**) in vitro by their IFN-γ production or in vivo by (**G**) their serum cytokine responses and (**H**) proliferation in blood. *$P < 0.05$; **$P < 0.01$. Data are shown as mean ± SEM from 1 experiment with groups of 4 mice that has been repeated twice with similar results.

investigated whether or not the pathway of antigen uptake also influenced the induction of NKT cell help for the priming of adaptive T cell responses. For this purpose, receptor-deficient and WT mice were immunized with suboptimal doses of OVA in combination with αGalCer as an adjuvant, and the primed OVA-specific CD4⁺ and CD8⁺ T cell responses were enumerated in spleen and lymph nodes by using MHC class I and II tetramers at day 8 after vaccination. Mirroring the limited contribution of LDLR uptake to αGalCer presentation in vitro (Figure 1A and Figure 3B), the

absence of the LDLR in vivo did not have a significant impact on the ability of αGalCer immunization to prime NKT cell help in vivo for the downstream expansion of CD4⁺ and CD8⁺ T cells (Figure 7G). In contrast, *Sra⁻/⁻* mice failed to mount OVA-specific CD4⁺ and CD8⁺ T cell responses, indicating that the NKT cell–mediated adjuvanticity was almost entirely abrogated in the absence of the SRA (Figure 7H). This suggested that for αGalCer, a hierarchy of receptor uptake pathways existed in vivo and demonstrated that the bioactivity of αGalCer was mostly attributable to SRA uptake.
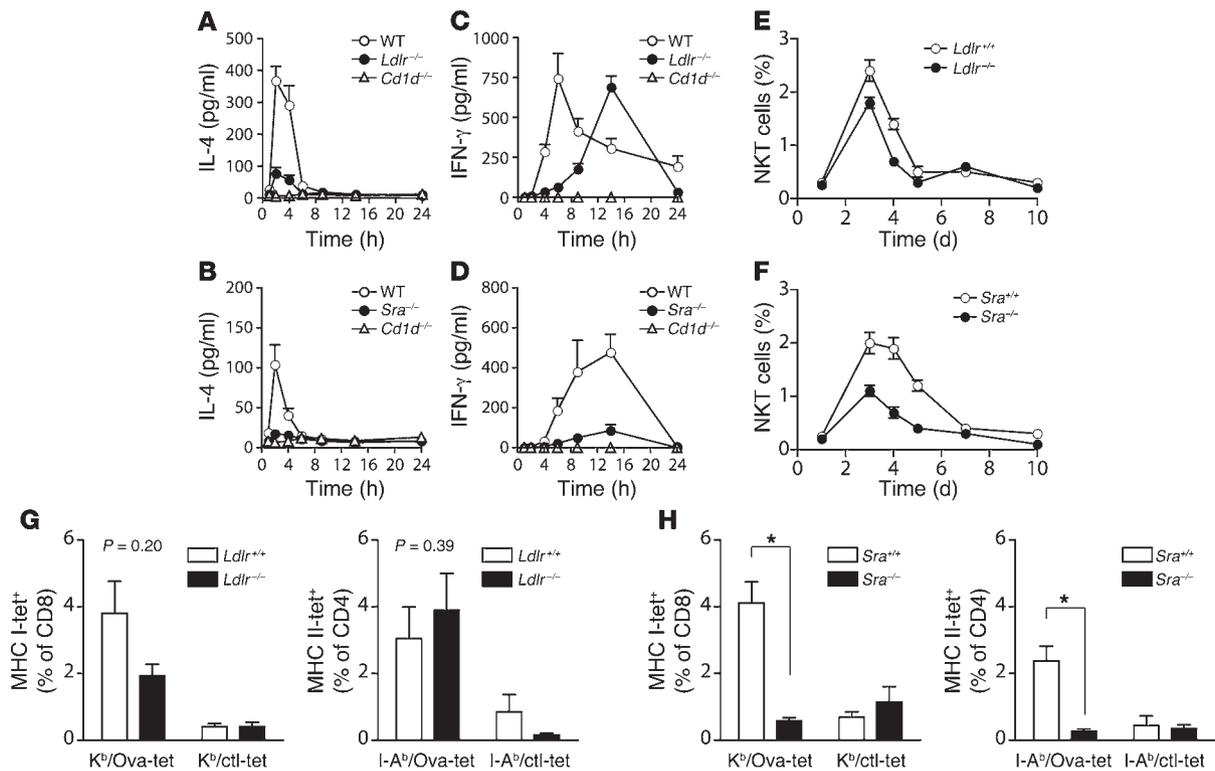
Figure 7
The class A SR SRA controls the bioactivity of αGalCer in vivo. (**A–D**) Serum cytokine responses upon αGalCer injection in the indicated receptor-deficient (black circles) and WT control mice (white circles). CD1d-deficient mice served as negative controls (white triangles). (**E** and **F**) Expansion of NKT cells of receptor-deficient (black circles) and WT control mice (white circles) in response to i.v. injection of αGalCer as assessed by CD1d tetramer staining of blood lymphocytes. (**G** and **H**) NKT cell help for the priming of OVA-specific CD4+ and CD8+ T cell responses in αGalCer/OVA-immunized *Ldlr*−/− mice but not in *Sra*−/− mice. Receptor-deficient mice (black bars) and their respective WT control mice (white bars) were immunized with OVA and αGalCer, and OVA-specific CD8+ and CD4+ T cell responses were evaluated on day 8 after immunization by staining of splenocytes with OVA-specific MHC class I and II tetramers. Irrelevant influenza hemagglutinin-specific tetramers served as negative control. Data of 3 (**A–F**) and 2 (**G** and **H**) independent experiments using groups of 4 mice are shown as mean ± SEM. *$P < 0.05$.

As these results identified SRA as the uptake receptor for αGalCer, we sought to further explore the principles governing lipid uptake for CD1 presentation in vivo. We evaluated the contribution of SRA and LDLR to the priming of individual NKT cell effector functions using 3 prototypic NKT cell agonists, each representing a distinct group with unique receptor uptake properties: PBS-44, which comparably depends on both receptors; PBS-29, an exclusively SRA-dependent lipid; and PBS-18, an exclusively LDLR-dependent agonist (Figure 8). These studies not only recapitulated the receptor requirements we had identified earlier in vitro (Figures 1 and 3), but also highlighted that the induction of individual NKT cell effector functions may require different efficiencies of lipid antigen uptake (Figure 8). For example, whereas immunization with PBS-18 or PBS-29 activated NKT cells to secrete IL-4 and IFN-γ and to proliferate in WT mice, these responses were strongly reduced in mice lacking the respective uptake receptors for PBS-29 (the SRA) or PBS-18 (the LDLR) (Figure 8, A–C). Furthermore, PBS-44 immunization of *Sra*−/− and *Ldlr*−/− mice resulted in WT levels of IFN-γ and normal NKT cell expansion, yet mice lacking either receptor failed to mount a functional IL-4 response. This suggested that both receptors contributed to the uptake of PBS-44 in vivo, but only partially compensated for each other (Figure 8). Thus, the extent of PBS-44 uptake achieved by one of the 2 receptors alone sufficed to trigger IFN-γ

and NKT cell proliferation, but failed to prime physiological IL-4 responses. A similar additive effect of the 2 uptake pathways could also explain the selective requirement for the LDLR in the αGalCer-induced IL-4 response in vivo (Figure 7A), whereas uptake of αGalCer in general was dominated by the SRA (Figure 1, Figure 3C, and Figure 7A). In comparison, efficient uptake of PBS-29 or PBS-18 depended on a single receptor, and absence of this receptor blunted the entire response in vitro (Figures 1 and 3) and in vivo (Figure 8). Similar to the NKT cell expansion and IFN-γ secretion, the adjuvant effect of PBS-44 vaccination for priming of adaptive CD8+ T cell responses could be mediated by either receptor (Figure 8, D and E). However, the adjuvanticity of NKT cell activation by PBS-29 or PBS-18 was negligible in WT mice (Figure 8, D and E), and we could not examine this response using these lipids.

These results confirmed in vivo that defined structural characteristics of lipid antigens determine their uptake pathways for CD1 presentation and indicated that the induction of individual NKT cell effector functions may require different efficiencies and thresholds of antigen uptake.

Discussion
NKT cell activation has emerged as a promising strategy for the immunotherapy of cancer, and great effort is being committed
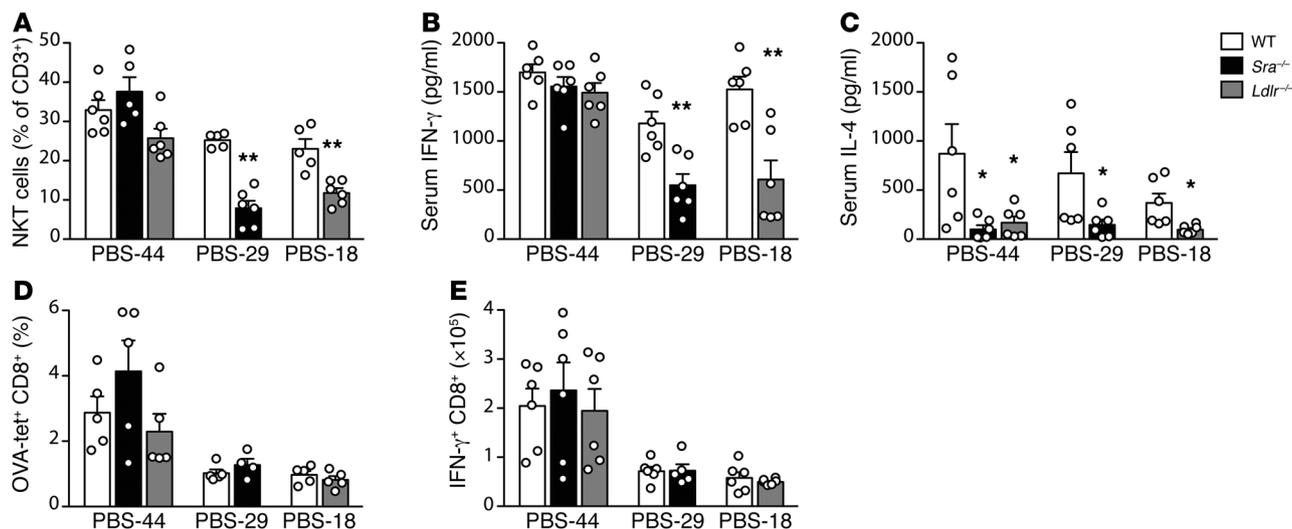
Figure 8
Differential contribution of individual uptake pathways to NKT cell responses in vivo. (**A–E**) In vivo NKT cell responses of immunized WT (white bars), *Sra*⁻/⁻ (black bars), and *Ldlr*⁻/⁻ (gray bars) mice in response to i.v. injection of indicated lipid antigens as assessed (**A**) by CD1d tetramer staining of blood lymphocytes on day 3 after infection and (**B**) serum IFN-γ and (**C**) serum IL-4 responses determined at 11 and 3 hours after immunization, respectively. (**D** and **E**) NKT cell help for the priming of OVA-specific CD8⁺ T cell responses in mice immunized with OVA and indicated NKT cell agonists. OVA-specific CD8⁺ responses were evaluated on day 8 after immunization by (**D**) staining of lymphocytes with OVA-specific MHC class I tetramers and (**E**) intracellular cytokine staining after restimulation with specific peptide. Circles depict individual mice, and bars represent the mean ± SEM for groups of 5–6 mice. *$P < 0.05$; **$P < 0.01$.

to developing NKT cell agonists into safe and powerful vaccine adjuvants. However, our knowledge of the transport and uptake pathways that direct lipid antigens for CD1d presentation in vivo remains very limited. In this study, we have identified SR-mediated uptake pathways as important contributors to CD1d antigen presentation and demonstrated that the in vivo bioactivity of the clinically relevant NKT cell agonist αGalCer was mainly controlled by uptake via SRA. Our findings suggest that the receptor targeting of lipid antigens may influence the resulting NKT cell response and characterize SRs as innate immune receptors that not only contribute to the recognition and clearance of pathogens, but also mediate the selection of lipid antigens for CD1d antigen presentation.

Due to their inherent hydrophobicity, lipids usually require the binding to proteins or lipoproteins for their transport in serum or extracellular milieu. Recently, a pivotal role of the VLDL fraction and its apoE has been proposed for targeting of lipids into the CD1d presentation pathway. In particular, it was demonstrated that CD1d presentation of the nominal NKT cell agonist digalactosylceramide critically depends on its apoE-mediated uptake via the LDLR (18). However, in view of our findings, it now appears that the uptake of individual lipids for CD1d presentation is mediated by a variety of different receptors, including the LDLR, SRA, SRB1, and CD36. Most interestingly, it appears that the specificity of this uptake is largely defined by the chemical structure of the lipid antigen rather than by the nature of the carrier vehicle used for transport. However, our data illustrated that very subtle differences were capable of dramatically altering the antigen uptake pathway. The interplay between LDLR and SRA uptake induced by modifications of either the head group (PBS-14, PBS-29) or acyl chain (PBS-44, PBS-57) are 2 of the best examples that we present here to argue that the chemistry of NKT cell agonists defines their biological profiles. Our experiments revealed that several receptors

with different ligand specificities contributed to antigen uptake for CD1d presentation, which suggests that an additional layer of specificity exists in the selection of lipids for CD1d antigen presentation. The availability of lipid antigens for CD1d presentation is primarily determined by the specificity of the lysosomal glycosidases (35) and lipid transfer proteins (41, 42) involved in the processing and loading of lipids into CD1d and by factors controlling lipid trafficking to the intracellular compartments where antigen processing and loading take place (43–45). The highly specific recognition of individual lipids by cell-surface receptors indicates that an additional level of antigen selection is imposed by receptor uptake. Whether the requirement for a given receptor pathway merely represents an initial recognition and binding step that determines the efficiency of antigen uptake or rather reflects the ability of the respective receptor to deliver the lipid toward the CD1d-loading compartment remains to be clarified.

Although SRs, such as the SRA, may focus the lipids targeted for CD1d presentation toward certain structural motifs, e.g., the presence of a negative charge, they also considerably broaden the range of antigens available for CD1 presentation. SRs are innate immune receptors that recognize pathogen-associated molecular patterns (PAMPs) and are involved in phagocytosis of Gram-negative as well as Gram-positive bacteria, or of stressed or apoptotic cells, oxidized lipoproteins, heat shock proteins, or polyanionic molecules in general. Notably, a link between SRA-mediated uptake and MHC antigen presentation similar to that observed for CD1d in our study has already been documented for the presentation of peptide antigens to MHC class I– and II–restricted T cells. In particular, deliberate targeting of antigen to SRs increased the induction of adaptive CD8⁺ and CD4⁺ T cell responses (33, 34). In addition, DCs have been shown to use SRA to acquire antigenic material from live cells for MHC class I cross-presentation (46).

Similarly, oxidative modifications that target lipoproteins toward SR uptake also greatly enhance the ability of such preparations to induce lipid-specific B cell responses (47). The linkage of SR-mediated uptake to CD1 antigen presentation shows that, in addition to apoE/LDLR bound lipids, a large family of lipids delivered by SR can access endocytic CD1-loading compartments, expanding the repertoire of antigens for CD1d presentation to an array of disease-relevant immunological targets, such as bacteria, infected or apoptotic cells, and modified lipoproteins.

Using the prototypic NKT cell agonist αGalCer, we compared the relative contribution of both receptor pathways for the physiological NKT cell activation in vivo. Surprisingly, although presentation of αGalCer to NKT cells in vitro seemed to involve both the SRA and the LDLR, the requirement for these uptake pathways to the activation of NKT cell responses very much differed in vivo. While $Ldlr^{-/-}$ mice exhibited only a reduced IL-4 production, but otherwise responded normally to αGalCer stimulation, absence of the SRA-mediated uptake pathway completely abrogated the NKT cell cytokine response as well as the adjuvant effect of NKT cell activation for the priming of adaptive T cells. These findings clearly demonstrated a hierarchy of both receptors in the response to αGalCer and identified SRA as the main uptake receptor for NKT cell activation by αGalCer. Nevertheless, the SRA and LDLR pathways compensated each other for all aspects of the NKT cell response to PBS-44, except IL-4, which appeared to require a very rapid efficient lipid uptake and a threshold of uptake of the agonist for secretion. In contrast, the uptake of agonists such as PBS-29 and PBS-18 was mediated by a single receptor, and the NKT cell response to these lipids critically depended on expression of this receptor in vivo. Importantly, we show that the contribution of individual receptor pathways to lipid uptake is dictated by the lipid antigen structure in vitro and in vivo. Given that our studies defined first structural characteristics that directed lipid antigens for preferential uptake via the LDLR or the SRA (Figures 1 and 3), it is conceivable that such "targeting motifs" could be applied to deliberately targeting NKT cell agonists toward specific uptake pathways in order to modulate the NKT cell response. Furthermore, the relative availability of SRs on APCs or the regulation of cellular SR expression in the context of infectious or inflammatory processes may directly influence the amplitude and quality of NKT cell responses induced by αGalCer and could therefore eventually determine the outcome of NKT cell vaccination.

Moreover, the differential NKT cell responses induced by lipid uptake via the LDLR versus the SRA pathways could also be relevant to the pathogenesis of atherosclerosis, a chronic inflammatory process of the vasculature to which NKT cells have been shown to contribute (48). Atherogenesis is characterized by increased oxidative stress and the presence of oxidatively modified lipoproteins, which result in a switch from LDLR-mediated toward SR-mediated uptake of VLDL/LDL particles. It is tempting to speculate that due to the increased uptake of oxidized lipoproteins via the SRA, lipoprotein particle–associated lipids, e.g., lysophospholipids (49), might get CD1 presented in a completely different context and consequently induce a different NKT cell effector response, which in turn may contribute to the pathogenic process.

In conclusion, our results delineate the important contribution of SR-mediated uptake pathways to the selection of lipid antigens for CD1d presentation and suggest that targeting of lipid antigens toward this uptake pathway could be used as an approach to enhancing NKT cell activation in therapeutic vaccination.

Methods

Mice. $Ldlr^{-/-}$ mice (N13 on the C57BL/6 background) (50) were originally obtained from L. Curtiss (TSRI). $Sra^{-/-}$ mice (30) were obtained from W. de Villiers (University of Kentucky, Lexington, Kentucky, USA) and from the Jackson Laboratory (N12 on the C57BL/6 background). $Cd36^{-/-}$ mice (28) were provided by K. Hoebe and B. Beutler (TSRI). SRB1-deficient mice (51) were bought from the Jackson Laboratory. Mice were bred locally at TSRI under specific pathogen–free conditions.

Reagents. Lipid antigens were synthesized as published previously (37, 38, 40). Lipid stocks (1 mg/ml in dimethylsulfoxide) were diluted to 0.2 mg/ml with PBS containing 0.02% Tween 20. Further dilutions were prepared in PBS for i.v. injections and in cell-culture medium for in vitro stimulation assays. All other reagents were from Sigma-Aldrich unless otherwise stated. Murine CD1d was produced and loaded with lipid antigen as published (40).

Antigen presentation assay. CD1d presentation of lipid antigens was detected using the murine NKT cell hybridoma DN32.D3, which expressed the semi-invariant Vα14Jα18/Vβ8 TCR. Hybridoma cells were cultured in RPMI supplemented with 10% FCS, 2 mM L-glutamine, 20 mM HEPES, and nonessential amino acids. DCs from WT or receptor-deficient mice were generated in in vitro bone marrow cultures in the presence of 2 ng/ml recombinant GM-CSF (Biosource; Invitrogen). Expression levels of LDLR and different SRs on WT and receptor-deficient DCs were confirmed by quantitative RT-PCR (qRT-PCR) (Supplemental Figure 2). For stimulation experiments, DCs were purified over a 14.5% Histodenz density gradient and then pulsed with titrated amounts of lipid antigens for 4 hours at 37°C. Cells were extensively washed, and 10^4 DCs were used to stimulate 5×10^4 DN32 cells per well in 96-well tissue culture plates. Cell-culture supernatant was collected 24 hours later for determination of IL-2 concentrations.

For blocking experiments, the lipid pulse of DCs was performed under serum-free conditions. In brief, bone marrow–derived DCs were harvested, washed 3 times with serum-free medium (containing 5% fatty acid–free BSA), and then plated at 10^4 cells per well in round-bottom 96-well tissue culture plates. Cells were incubated for 30 minutes at 37°C in serum-free medium before titrated amounts of lipoproteins (or SR ligands) were added and the incubation was continued for an additional 30 minutes to saturate the lipoprotein uptake receptors. Next, titrated amounts of lipid antigens (in serum-free medium) were added to the receptor-blocked DCs. After a further 60-minute pulse with lipid antigen in the presence of lipoproteins, the DCs were washed (twice with PBS, followed by a wash with culture medium containing 10% FCS) before 5×10^4 DN32 cells (in 100 μl culture medium with 10% FCS and additives) were added per well. Plates were incubated at 37°C, and cell-culture supernatant was collected 24 hours later.

Determination of cytokine concentrations. The IL-2 concentration in culture supernatants was determined using the [^{3}H]-thymidine incorporation of an IL-2–dependent cell line as readout. The IL-4 and IFN-γ concentrations in the serum of immunized mice were determined using BD OptEIA Elisa Sets (BD Biosciences — Pharmingen).

Isolation of mouse serum lipoproteins. Murine lipoproteins were isolated from the blood of healthy $Ldlr^{-/-}$ mice that had been fed a diet containing 1% cholesterol (TD95286; Harlan Teklad) for 4–6 weeks. Lipoproteins were freshly prepared for each experiment. Blood was obtained from the vena cava, and the VLDL ($d < 1.006$ g/ml, where d indicates density), LDL ($1.023 < d < 1.058$ g/ml) and HDL ($1.063 < d < 1.19$ g/ml) fractions were isolated by sequential ultracentrifugation using discontinuous NaBr density gradients as previously described (47). Lipoprotein fractions were stored under nitrogen and used within 14 days.

Lipid antigen distribution in murine serum. Mouse serum was pulsed with 1 μg/ml of individual lipid antigens at 37°C for 3 hours and then fractionated over a HiPrep 16/60 Sephacryl S-300 gel filtration chromatography column

(GE Healthcare) using an ÄKTAprime FPLC machine (GE Healthcare). Fractions (0.5 ml) were collected and sterile filtered (0.2 μm). The presence of lipid antigen was detected by DN32.D3 hybridoma stimulation in vitro using C57BL/6 splenocytes as APCs. Protein and cholesterol concentrations of individual fractions were determined with the BCA Protein Assay (Pierce) and the Cholesterol Infinity Reagent (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions.

Immunizations. Mice were immunized with αGalCer i.v. For adjuvant experiments, animals were i.v. injected with 100 ng αGalCer 30 minutes before receiving 250 μg OVA in a separate injection. Blood samples were collected at indicated times after immunization for determination of serum cytokine levels by ELISA and of expansion of NKT cells by staining with CD1d tetramers. The priming of OVA-specific CD8^{+} and CD4^{+} T cells was assessed in blood and spleen using MHC class I and II tetramers 8 days after immunization.

Flow cytometry. NKT cells were quantified in blood and organs using CD1d tetramers loaded with αGalCer. Tetramer and antibody staining were performed on blood collected from the retroorbital plexus or on single-cell suspensions prepared from spleens, livers, and thymi. Organs were collected in cold PBS, and spleens and thymi were passed through a 70-μm cell strainer to obtain a single-cell solution. Livers were first dissected to small pieces and then passed through a cell strainer before lymphocytes were isolated by Lympholyte M gradient centrifugation (Cedarlane Laboratories Ltd.). Single-cell solutions were treated with Fc Block (BD Biosciences) and 0.5 mg/ml avidin (Sigma-Aldrich) in FACS buffer (FB) (PBS containing 2% FCS/2 mM EDTA) at room temperature for 10 minutes. Cells were then washed with FB and stained with CD1d/αGalCer tetramers at room temperature for 15 minutes. Anti–CD3ε-APC, anti-B220 or anti-CD44 antibodies (all from BD Biosciences — Pharmingen) were directly added, and staining continued for another 30 minutes. After 2 washes with FB, samples were depleted of erythrocytes and fixed with the BD FACS Lysing Solution (BD Biosciences). Samples were acquired on an LSR II system using FACSDiva software (both BD Biosciences) and analyzed with FlowJo software (Tree Star Inc.).

Statistics. Statistical significance was determined using the unpaired *t* test (Prism; GraphPad Software). $P < 0.05$ was considered significant.

Study approval. All experimental procedures involving animals were performed according to TSRI institutional guidelines and were approved by the TSRI Institutional Animal Care and Use Committee.

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