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Lysosomal processing of sulfatide analogues alters target NKT cell specificity and immune responses in cancer

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ABSTRACT

In a structure-function study of sulfatides, that typically stimulate type II NKT cells, we made an unexpected discovery. We compared analogues with sphingosine or phytosphingosine chains and 24-carbon acyl chains with 0-1-2 double bonds (C or pC24:0, 24:1, or 24:2). C24:1 and C24:2 sulfatide presented by CD1d monomer on plastic stimulated type II, not type I, NKT-cell hybridomas as expected. Unexpectedly, when presented by bone-marrow-derived DCs (BMDCs), C24:2 reversed specificity to stimulate type I, not type II, NKT-cell hybridomas, mimicking the corresponding βGalCer without sulfate. It induced IFNγ-dependent immunoprotection against CT26 colon-cancer lung metastases, skewed the cytokine profile, and activated cDC1s. This was abrogated by blocking lysosomal processing with bafilomycin A1, or sulfite-blocking or deletion of arylsulfatase A that cleaves off sulfate. Thus, C24:2 is unexpectedly processed in BMDCs from a type II to a type I NKT cell-stimulating ligand, promoting tumor immunity. We believe this is the first discovery of antigen processing of glycosylceramides altering the specificity for the target cell that reverses its function from stimulating type II to stimulating type I NKT cells, introducing protective functional activity in cancer. It also uncovers a new role for antigen processing, not to allow MHC loading but to alter the cell responding.

ONE SENTENCE SUMMARY

Unexpected lysosomal glycolipid processing reverses target NKT cell specificity, thereby altering anti-cancer function.

INTRODUCTION

The recent clinical success of checkpoint inhibitors, which manipulate T cell function and extend patient survival in many cases, provides strong motivation to treat cancer by modulating immunity. However, a certain large proportion of patients across multiple cancers still cannot benefit from the existing immunotherapies [1, 2]. Therefore, understanding the interactions between tumor development and the immune system and developing other novel immunotherapeutic approaches are urgently needed.

Natural Killer T (NKT) cells are a unique T cell subset that is developmentally and functionally distinct from conventional T cells. NKT cells develop in the thymus and express T cell receptors (TCRs) which recognize lipid antigens presented by the major histocompatibility complex (MHC)-like molecule, CD1d [3-7]. NKT cells then follow a subsequent development pathway and are detected in the periphery in a partially activated state, harboring pre-formed mRNA transcripts coding for several cytokines, allowing very rapid secretion of large amounts of cytokines upon stimulation. Thus, NKT cells serve as components of innate immunity and adaptive immunity and potentially can drive subsequent responses of other immune cells [8, 9].

There are two main subsets of NKT cells, termed type I and type II NKT cells. Type I NKT cells are characterized primarily based on their invariant TCRα expression (Vα24Jα18 in humans and Vα14Jα18 in mice), which is paired with a limited set of TCRβ chains, and reactivity to the glycolipid α-galactosylceramide (αGalCer) (which we will use to refer to

the specific structure of KRN7000 - see Fig. 1B). On the other hand, type II NKT cells express a different and more diverse TCR repertoire than type I NKT cells [4, 7]. The most widely studied antigen for type II NKT cells is sulfatide. Sulfatide-reactive type II NKT cells are reported to have immunosuppressive functions in experimental autoimmune encephalomyelitis [10], autoimmune hepatitis [11], type I diabetes [12], and allergic airway inflammation [13, 14]. In tumor settings, we previously reported that sulfatide-reactive type II NKT cells suppress tumor immunosurveillance in experiments showing that the injection of sulfatide increased the development of lung metastasis and inhibited the protective effect of type I NKT cells [15]. These suppressive activities of type II NKT cells were mainly reported to be induced by stimulating them with native sulfatide, a mixture of different sulfatide isoforms.

Although many studies have been published on structure-function relationships among αGalCer analogues that stimulate type I NKT cells, including variants that elicit distinct cytokine profiles (see Discussion) [16-24], the functional activities of each component of native sulfatide or synthetic sulfatide analogues have not been well described yet. This study aimed to investigate the functional activities of synthetic sulfatide analogues, especially against tumor immunity, and gain better knowledge about the structure-function relationship of sulfatides to develop novel strategies for anti-tumor immunotherapies. The analogues were produced by modifications of the number of double bonds in the acyl chain and the type of sphingoid base in the ceramide structure. Of importance is the discovery that the C24:2 analogue with two double bonds and a sphingosine base significantly reduced the development of lung metastases. In exploring the mechanisms of this structural effect on

104 function, we discovered that a major factor is how these molecules are processed by
105 dendritic cells (DCs). We found that C24:2 stimulates type I NKT cells when processed and
106 presented by DCs, whereas it stimulates type II NKT cells when presented by CD1d
107 monomer on plastic. Although some processing of glycolipid NKT agonists has been
108 described [21, 25], such processing of glycosylceramides to alter their specificity for
109 different target cells and thereby alter their function has not been observed previously to our
110 knowledge. We believe that understanding the mechanisms which underly the relationships
111 between sulfatide endosomal or lysosomal processing and NKT cell functions opens
112 promising new strategies for cancer immunotherapies.
113

RESULTS

Sulfatide analogues with either a sphingosine base or a phytosphingosine base stimulate type II NKT cells.

In this study, six different sulfatide analogues, of which four were newly synthesized (C24:2, pC24:0, pC24:1, and pC24:2,), were evaluated (Fig. 1A). These analogues are classified into two groups based on their sphingoid base, C18-sphingosine or C18-phytosphingosine [26], the latter being the sphingoid base of KRN7000, the prototypical agonist of type I NKT cells (Fig. 1B). Each sulfatide contains an acyl chain with 0, 1, or 2 double bonds. The sulfatides are designated C24:0, C24:1, and C24:2 for the sphingosine series and pC24:0, pC24:1, and pC24:2 for the phytosphingosine series (Fig. 1A). The sulfatide analogue, C24:1, is the major component of the native sulfatide mixture in the myelin of the nervous system. C24:1 was shown to be one of the immunodominant components of native sulfatide to stimulate type II NKT cells in previous reports [27-29]. First, we investigated the immunoreactivity of each sulfatide analogue utilizing the sulfatide-reactive CD1d-restricted NKT cell hybridoma clone, XV19, which was derived from type II NKT cells [30]. We stimulated XV19 with plate-bound mCD1d monomer loaded with each sulfatide analogue and measured IL-2 levels in the culture media as an activation marker. Titration curves of each analogue showed that, among the sphingosine base group, C24:1 showed the highest and C24:0 showed the lowest stimulation of XV19 (Fig. 1C, left), which is consistent with previous reports [27]. In the phytosphingosine base group, the corresponding analogues had a similar rank order of reactivity, with pC24:0 being the least potent and pC24:1 the most, but still weaker than C24:1 in both magnitude of

response and potency on a molar basis (Fig. 1C, right). These results suggest that all the sulfatide analogues tested stimulate type II NKT cells, and C24:1 induces the highest immunoreactivity among the six analogues used in this assay.

C24:1 and C24:2 stimulate type II NKT cells specifically in a CD1d-dependent manner.

Next, to better characterize each sulfatide analogue, we stimulated both type I and type II NKT cell hybridoma clones (DN32 and XV19, respectively) with each sulfatide analogue at the same concentration (0.5 μM), presented by plate-bound CD1d monomer (Fig. 2A). Notably, the maximum level of IL-2 secreted from DN32 and XV19 in response to anti-CD3 is significantly different. Likewise, the absolute magnitude of the response to lipids varied between the hybridoma clones; so the magnitudes must be compared only in relation to the anti-CD3 control for that hybridoma, and not between hybridomas. All three analogues in the phytosphingosine base group activated both type I and type II NKT cell hybridoma clones, although they stimulated the type II NKT cell hybridoma more strongly than the type I NKT cell hybridoma. It is not clear why the analogues with a phytosphingosine base group activated type I NKT cells to a limited extent. Clearly, the way the sphingosine and acyl chains fit into pockets in the CD1d molecule can affect T cell specificity even though these are not exposed, but could influence the orientation of the exposed portion. KRN7000 also has a phytosphingosine base, but it is hard to make a structural connection without crystallography or other molecular imaging techniques. In contrast, C24:2 with a sphingosine base, as well as C24:1, activated only the type II NKT cell hybridoma, but not the type I NKT cell hybridoma. Therefore, these analogues are type

II NKT cell-specific. C24:0 activated neither of the hybridoma clones. We also confirmed that the stimulation by sulfatide analogues is CD1d-dependent because the presence of anti-CD1d antibody (clone 20H2) completely abolished the reactivity of the sulfatide analogues. In contrast, the stimulation by anti-CD3 antibody (Fig. 2A) was not affected. Since C24:1 and C24:2 were specific ligands for the type II NKT cell hybridoma, but not stimulatory for the type I NKT cell hybridoma DN32, we decided to focus on them for further investigation.

C24:1 and C24:2 were titrated with the type I NKT cell hybridoma clone, DN32. Neither of these compounds stimulated this type I NKT cell hybridoma at any concentration ranging from 0.1 to 30 μM presented by CD1d monomer on plastic (Fig. 2B, left). At the same time, we again confirmed that C24:1 and C24:2 stimulated the type II NKT cell hybridoma clone, XV19, with a bell-shaped titration curve (Fig. 2B, right). In addition, we stimulated two other type I NKT cell hybridomas, 24.9E and 24.8A, with sulfatide analogues to confirm that these analogues do not stimulate at least three different hybridoma clones of type I NKT cells. 24.9E and 24.8A are type I NKT cell hybridoma clones that differ from DN32, by their Vβ/Jβ gene rearrangements (DN32: Vβ8.2/Jβ2.4, 24.9E: Vβ8.3/Jβ2.4, and 24.8A: Vβ8.2/Jβ2.5). They react to different kinds of ligands (e.g., 24.8A reacts more with phosphatidyl-inositol than with αGalCer) and have different magnitudes of reactivity even to the same ligand [31]. As shown in Fig. 2C, C24:1 and C24:2 did not stimulate either 24.9E or 24.8A. Although KRN7000 did not stimulate 24.8A either, this is consistent with a previous report [31]. Thus, these sulfatide analogues presented by CD1d monomers on plastic do not stimulate any of three type I NKT cell hybridoma clones we tested.

The effects of sulfatide analogues on tumor immunity.

We previously reported that *in vivo* injection of native sulfatide in a murine model of lung metastasis increased the number of lung nodules, whereas KRN7000 reduced it [15]. Using the same model, we tested the impact of the sulfatide analogues on the establishment of lung metastases. CT26 tumor cells were injected intravenously (i.v.) in wild-type (WT) mice, which subsequently received a single dose of lipid injection intraperitoneally. C24:1, which has been reported to be the immunodominant component of native sulfatide, had no significant impact on the number of lung metastasis compared to the vehicle-injected group, but C24:2 significantly reduced the development of lung metastases in a dose-dependent manner (Fig. 3A and 3B), albeit not as completely as KRN7000. The unexpected protection by C24:2 could theoretically have been due to either stimulation of an altered functional response of type II NKT cells or stimulation of type I NKT cells.

C24:2 promotes tumor immunity through an IFNγ-dependent mechanism.

To further investigate the mechanism explaining the different outcomes with C24:1 and C24:2 *in vivo,* we conducted an *ex vivo* study. We isolated spleen mononuclear cells (MNCs) from WT mice, stimulated with each sulfatide analogue and analyzed cytokine production. Spleen MNCs from WT mice stimulated with C24:2 produced a higher amount of both Th1 and Th2 cytokines compared to C24:1, and this cytokine production was CD1-/NKT cell-dependent, as spleen MNCs from *Cd1*-deficient (*Cd1*-KO) mice did not produce any cytokine when stimulated with either lipid (Fig. 4A). In addition, we examined lung MNCs, as the lung is the site at which the i.v. injected CT26 cells are trapped and form the tumor nodules. Similar to the results of the spleen cells, lung MNCs incubated with C24:2-

pulsed BMDCs produced a higher amount of both Th1 and Th2 cytokines compared to lung MNCs stimulated with C24:1-pulsed BMDCs (Fig. 4B). We also confirmed *in vivo* that injection of C24:2 stimulated much more cytokine production as measured in plasma than C24:1 in WT mice (Fig. 4C and 4D). Thus, the increased cytokine production was not limited to an *in vitro* observation. In addition, we analyzed the serum cytokine levels of mice injected with each lipid across different time points (3 h, 6 h, 12 h, and 24 h after injection). The heat map of individual cytokine levels in serum showed a pattern similar to that in plasma (Supplementary Fig. S1). The principal component analysis (PCA) data generated from the same experiment demonstrated that the clusters of KRN7000-, C24:1-, and C24:2-injected mice were distinct from each other (Fig. 4E). As the cytokine production in plasma stimulated with C24:2 was more Th1 skewed than that stimulated with C24:1, because both ratios of IFNγ/IL-4 and IFNγ/IL-13 were higher in C24:2 (Fig. 4F), we hypothesized that the difference between the effects of C24:1 and C24:2 in tumor immunity was dependent on IFNγ production. To address that, we injected each lipid into IFNγ-deficient (*Ifng*-KO) mice that had been previously injected with CT26 cells and counted lung metastasis nodules. Consistent with our hypothesis, in the absence of IFNγ, C24:2 did not reduce the number of lung metastasis (Fig. 4G, no statistically significant differences across all groups), in contrast to that seen in WT mice (Fig. 3A). These data suggest that C24:2 promotes tumor immunity through an IFNγ-dependent mechanism.

Since we observed anti-tumor function from C24:2 in the CT26 tumor metastasis mouse model, we further verified that C24:2 was not potentially contaminated with a variant form that would be more immunostimulatory, such as the alpha anomer of C24:2. To address this, we generated the alpha anomer of C24:2 (SR-22-24A), and the alpha anomer of C24:1

(αC24:1) (Supplementary Fig. S3A). We titrated SR-22-24A and C24:2 with BMDCs to activate DN32 and saw a 1,000-fold greater reactivity to the alpha anomer (Supplementary Fig. S3B). Additionally, we titrated SR-22-24A and αC24:1 with CD1d-lipid complexes on plastic and compared them to their beta anomers and observed differing reactivity to activation of DN32 (Supplementary Fig. S3C). Although we could not assess the magnitude of the difference in reactivity for C24:2 compounds because the beta anomer did not stimulate, we observed that αC24:1 had a least a 1,000-fold difference in reactivity compared to C24:1. Since these compounds all underwent similar synthesis processes, we believe that any potential alpha anomer contamination would have to be under 0.1% or it would have been detected in the hybridoma assays without processing.

The difference of antigen-presenting cells (APCs) and co-signaling molecules expressed on APCs between C24:1 and C24:2 injected mice.

In our *in vivo* experiment, the plasma cytokine production of mice injected with sulfatide analogues showed that not only IFNγ but also other cytokines, including IL-12p70 and sCD40L, were significantly higher in C24:2-injected mice compared to C24:1-injected mice (Fig. 4C and 4D). These results indicate that the interaction of NKT cells and APCs might be involved in the different outcomes of tumor immunity between C24:1 and C24:2. NKT cells get activated through the recognition of glycolipids presented by CD1d-expressing APCs without affecting their CD1d expression level (Supplementary Fig. S2). Also, further outcomes of cellular interactions during immune responses are controlled by co-signaling molecules expressed on the cell surface of APCs. Therefore, to gain more insight into how C24:2 promotes tumor immunity through an IFNγ-dependent mechanism, we investigated

252 the difference in phenotypes of APCs and their co-signaling molecules between the mice
253 injected with C24:1 or C24:2. We injected WT mice with each lipid and after 24 h harvested
254 and analyzed their spleen MNCs stained with monoclonal antibodies specific for cell surface
255 markers and co-signaling molecules. As shown in Fig. 5B, the number of conventional DCs
256 (cDCs) (CD11c+B220–, gating shown in Fig. 5A) was significantly greater in C24:2-
257 injected mice than in C24:1-injected mice, while other APCs (plasmacytoid DCs (pDCs),
258 B220+CD11c+; B cells, B220+ TCRβ–; CD11b+ cells, B220–TCRβ–CD11c–CD11b+, Fig.
259 5A) did not display any significant differences between mice injected with C24:1 or C24:2.
260 Since there are mainly two subsets within cDCs, termed cDC1 and cDC2, we next
261 investigated these two subsets. The CD8α+ CD11b– population, which represents cDC1s
262 (cross-presenting DCs), has been reported to be important in anti-tumor immune responses
263 [32-34]. We found that within the cDCs, this cDC1 population was significantly increased
264 in C24:2-injected mice compared to C24:1-injected mice (Fig. 5C), although a trend was
265 also seen for more cDC2 cells. Moreover, among the co-signaling molecules of cDC1s, the
266 MFI of CD80 was significantly higher in C24:2-injected mice than in C24:1-injected mice
267 (Fig. 5D). In contrast, it is worth noting that C24:1 seemed to induce little or no changes in
268 APC populations. These results suggest that C24:2, not C24:1, induces cDC (especially
269 cDC1) expansion and higher expression of co-stimulating molecules and, therefore, induces
270 subsequent immune responses, which result in the enhancement of the anti-tumor immunity.
271 These cells may be the source of higher levels of IL-12, which could in turn induce higher
272 levels of IFNγ.
273

Sulfatide-pulsed BMDCs stimulate the type I NKT cell hybridoma, but not the type II NKT cell hybridoma, dependent on lysosomal acidification.

As we showed that *in vivo* C24:2 injection induced expansion and activation of cDC1 cells, we next stimulated hybridoma clones using BMDCs as APCs to present sulfatide analogues *in vitro*. We pulsed BMDCs with each lipid, washed, and incubated with hybridoma clones, and measured IL-2 production in the culture media. IL-2 levels produced by XV19 using BMDCs pulsed with the vehicle, C24:1, C24:2, or KRN7000 were the same background level (Fig. 6A, left), indicating a lack of specific response to the lipids. On the other hand, DN32 was stimulated by C24:1-, C24:2-, or KRN7000-pulsed BMDCs, and those stimulations were CD1d-dependent as the stimulation was blocked by anti-CD1d antibody, 20H2 (Fig. 6A, right). Opposite to the results of the assays stimulating with cell-free CD1d monomer loaded on the plates shown in Fig. 2, these results showed that sulfatide analogues pulsed on BMDCs stimulate the type I NKT cell hybridoma but not the type II NKT cell hybridoma.

It is known that sulfatides are hydrolyzed to cleave the sulfate moiety in lysosomes and become the corresponding β-galactosylceramides (βGalCers) [35]. Thus, we speculated that BMDCs internalize the sulfatides, and they are processed and degraded by lysosomal enzymes into βGalCers, which in turn stimulate type I NKT cells. To test that hypothesis, we used the lysosomal acidification inhibitor, bafilomycin A1, to inhibit the degradation of sulfatide in lysosomes. As shown in Fig. 6C, bafilomycin A1 markedly reduced IL-2 production from DN32 stimulated with sulfatide-pulsed BMDCs. A similar pattern was observed in another type I hybridoma clone, 24.9E cells (Fig. 6D). These results suggest that the degradation in lysosomes is necessary for sulfatides to stimulate the type I NKT cell

hybridoma, and degraded sulfatides do not stimulate the type II NKT cell hybridoma (Fig. 6B). The fact that the bafilomycin A1 inhibition of lysosomal processing does not restore stimulation of the type II NKT cell hybridoma (Fig. 6B) suggests that loading of sulfatide takes place primarily in acidified lysosomes. Indeed, we and others have previously observed that loading sulfatide onto free CD1d monomers *in vitro* requires low pH and lipid transfer proteins as a catalyst [36]. Hence, the bafilomycin A1 also prevents this loading of the unprocessed sulfatide which requires endosomal loading but not surface loading at neutral pH. Note that bafilomycin A1 does not inhibit stimulation by anti-CD3 as a control for non-specific inhibition or toxicity.

βGalCer analogues that result from sulfatase cleavage of the corresponding sulfatides are more potent type I NKT cell stimulators.

Although β-linked GalCer analogues had not been found to be very strong stimulators of type I NKT cells or to have potent anti-tumor activity, these βGalCer C24:1 and βGalCer C24:2 analogues had never been tested. We, therefore, compared sulfatides and their corresponding βGalCer versions both *in vitro* and *in vivo*. Fig. 7A shows that plate-bound CD1d monomer loaded with sulfatides stimulate only the type II NKT cell hybridoma, as seen in Fig. 2 above, and plate-bound CD1d loaded with βGalCers stimulate only the type I NKT cell hybridoma. On the other hand, Fig. 7B shows that BMDCs pulsed with either sulfatides or βGalCers fail to stimulate the type II NKT cell hybridoma, but both sulfatides and βGalCers stimulate the type I NKT cell hybridoma. Fig. 7B also shows that when the lipids are pulsed onto BMDCs and presented to the type I NKT cell hybridoma at the same concentration, IL-2 production by βGalCers is higher than with the corresponding sulfatides.

To rule out contamination of the βGalCers with the corresponding alpha anomers, we synthesized and tested these. Supplementary Fig. S3B shows that the alpha anomer of βGalCer C24:2 is about 100-fold more potent on a molar basis to stimulate DN32, but about 10-fold less potent than KRN7000. There is no way that contamination with KRN7000 could have occurred as it is not even present in the lab that did the synthesis. Chemical tests are not sensitive enough to exclude a 0.1% contamination of βGalCer C24:2 with its alpha anomer, but the synthetic method used makes such a contamination extremely unlikely. In Supplementary Fig. S3C, we also show a titration comparing the sulfatides C24:1 and C24:2 with their alpha anomers (with sulfate) to stimulate DN32 when coated onto CD1d monomers on plastic. Since the alpha anomers stimulate but the beta anomers do not at the highest concentration testable, we think these sulfatides could not be contaminated with their alpha anomers either. The same trends were observed in an *in vivo* mouse tumor challenge experiment (Fig. 7C).

To further confirm that the processing of C24:2 sulfatide in DC lysosomes was mediated by the known enzyme arylsulfatase A to cleave the sulfate moiety, we examined the effect of sulfite inhibition of arylsulfatase A, as has been previously described [37]. Results show that titrated amounts of sulfite reduce the stimulation of DN32 by DCs pulsed with C24:2 in a dose-dependent manner, without affecting stimulation by anti-CD3 and KRN7000, as a control for nonspecific toxicity within this concentration range (Fig. 8A, left). Moreover, stimulation by βGalCer C24:2 is not affected by the inhibition of arylsulfatase A, as the presentation of βGalCer C24:2 does not require arylsulfatase A. A parallel titration of bafilomycin A1 was also carried out (Fig. 8A, right), showing similar specific inhibition of

343 stimulation by C24:2 without any effect on KRN7000, βGalCer C24:2, or anti-CD3, as a
344 control for nonspecific toxicity or other inhibitory effects over this concentration range. In
345 addition, to confirm the role of arylsulfatase A in BMDC to process C24:2, we utilized
346 arylsulfatase A-deficient mice (*Arsa*-KO). Indeed, BMDCs generated from *Arsa*-KO mice
347 failed to process and present C24:2 and activate DN32 cells compared to the BMDCs
348 generated from WT mice (Fig. 8B).
349
350 We also hypothesized that if cleavage to βGalCer C24:2 was the mechanism by which DCs
351 pulsed with C24:2 stimulate type I NKT cells, then we should detect activation of type I
352 NKT cells *in vivo*, and the protection should be dependent on type I NKT cells as well. To
353 test these, first we looked at the induction of expression of the early activation marker CD69
354 on PBS57-loaded CD1d-tetramer positive type I NKT cells at 12 h after injection of C24:2
355 *in vivo* (Fig. 8C). Indeed, CD69 was upregulated on type I NKT cells significantly
356 compared to the vehicle control and the C24:1 sulfatide, albeit not as much as induced by
357 KRN7000. To test dependence on type I NKT cells, we used *Traj18*-KO mice that lack type
358 I NKT cells but retain type II NKT cells. As predicted, no tumor protection was induced by
359 C24:2 in *Traj18*-KO mice (Fig. 8D) (Note that the absence of type I NKT cells in *Traj18*-
360 KO mice does not affect CD1d expression) [38].
361
362 These results were further confirmed and shown to be translatable to humans in primary
363 human peripheral blood mononuclear cells (PBMCs). C24:2 stimulation of human PBMC
364 induced production of IFNγ in type I NKT cells defined as CD3+ PBS57-loaded CD1d
365 tetramer+ (Supplementary Fig. S4 and Fig 8E). Notice that the stimulation by C24:2 of

366 human type I NKT cells was inhibited by bafilomycin A1, showing that the processing we
367 described in the mouse translates in human NKT cells as well. This concentration of
368 bafilomycin A1 was not toxic to human NKT cells, as we show in the right panel of Fig. 8E
369 that it does not affect their activation by Cell Activation Cocktail (PMA and Ionomycin),
370 which does not require processing.
371 We conclude that tumor protection is mediated by the stimulation of protective type I NKT
372 cells by the processed sulfatide glycolipid, a phenomenon not previously described. This
373 basic phenomenon of processing sulfatides in endosomes to change the cellular specificity
374 of the lipid translates to human type I NKT cells as well.
375

DISCUSSION/CONCLUSION

In this study, we demonstrated that the newly synthesized sulfatide analogue C24:2 had a potent anti-tumor effect induced through an IFNγ-dependent mechanism, distinct from the biological effect of C24:1 that differs by only one double bond. We found unexpectedly that, when sulfatide analogues, C24:1 and C24:2, were presented by DCs, they lost their ability to stimulate a type II NKT cell hybridoma and gained stimulation of several type I NKT cell hybridomas. This was abrogated by bafilomycin A1, which blocks endosomal/lysosomal processing, and by blockade (or knock out) of arylsulfatase A that can cleave the sulfate moiety, suggesting that the tumor protection afforded by C24:2 *in vivo* may depend not on altered stimulation of type II NKT cells but on altered specificity for different target cells caused by lysosomal processing to stimulate type I NKT cells, in addition to inducing activation and expansion of cDC1 cells.

Yu K.O. *et al.* previously reported that altering the ceramide structure of KRN7000 by having a shortened, unsaturated fatty acid chain or having a sphingosine base instead of a phytosphingosine base could modify the functional property of type I NKT cells [20]. However, similar structure-function studies have not been available for type II NKT cell ligands. We sought to investigate whether altered structures of type II NKT cell ligands might produce altered functions that could potentially be used to overcome their known immunosuppressive activity and develop new therapeutic strategies for cancer immunotherapies. Our study demonstrated that, indeed, the immunoreactivity of type II NKT cells was also modified by altering the number of double bonds in a fatty acid chain or

399 of a sphingoid base group in the ceramide structure of sulfatide. Previous studies [27, 28]
400 showed that, among sulfatide isoforms that contain a fatty acid chain, C24:1 induced the
401 most potent stimulation of a type II NKT cell hybridoma. Consistent with these data, our
402 results also demonstrated that C24:1 induced the highest immunoreactivity of the type II
403 NKT cell hybridoma among the six sulfatide analogues we tested, of which four are newly
404 synthesized analogues. However, such titrations cannot distinguish the affinity for CD1d
405 from the affinity of the TCR for the CD1d-lipid complex. One of the strengths of our study
406 is that we also investigated the immunoreactivity of each sulfatide analogue by both type I
407 and type II NKT cells simultaneously presented either on CD1d monomer on plastic or
408 pulsed onto BMDCs. We confirmed that analogues with a sphingosine base stimulated type
409 II NKT cells specifically and exclusively when presented on CD1d on plastic. In contrast,
410 analogues with a phytosphingosine base stimulated both type I (albeit to a substantially
411 lesser extent) and type II NKT cells. Some stimulation of type I NKT cells by sulfatides
412 with a phytosphingosine base had been described before [39]. To provide more rigorous
413 evidence of the specificity of the sphingosine base group not to activate type I NKT cells,
414 we employed three different type I NKT cell hybridoma clones (DN32, 24.9E, and 24.8A)
415 which have distinct TCRs and different immunoreactivities and fine specificities. We found
416 that C24:1 and C24:2 presented by CD1d monomer on plastic induced no reactivity from
417 any of these type I NKT cell hybridoma clones. These results suggested that C24:1 and
418 C24:2 (sphingosine base) are both exclusive ligands for type II NKT cells, even though they
419 induce different functional properties of type II NKT cells.
420

421 Our current data demonstrated that sulfatide analogues that possessed a phytosphingosine
422 base also stimulated the type II NKT cell hybridoma. The phytosphingosine base group
423 induced immunoreactivity not only by the type II NKT cell hybridoma but also by the type I
424 NKT cell hybridomas. Wu D. *et al.* previously reported that a sulfatide analogue that
425 possessed a phytosphingosine base was stimulatory for human type I NKT cells [39]. This,
426 together with our data, suggests that the alteration of the structure of the sphingosine base to
427 the phytosphingosine base may play a role in the ability of these sulfatide analogues to
428 stimulate type I NKT cells. Further research is needed to provide an underlying mechanism
429 for these observations.
430
431 Previous reports also showed that sulfatide and its analogues did not stimulate the type I
432 NKT cells [10, 12]. Previous sulfatide analogue studies have mostly involved experiments
433 using hybridomas; so it was not possible to determine whether the different sulfatide
434 isoforms could modulate the cytokine profiles or disease outcomes. Our results above also
435 focused on NKT cell hybridomas and their reactivity to sulfatide analogues presented by
436 CD1d monomers coated onto the plastic culture plates. We developed our study toward *ex*
437 *vivo* and *in vivo* settings so that we could assess the interaction of sulfatide analogues with
438 primary mouse cells and effects on tumor growth. Surprisingly, a single *in vivo* injection of
439 C24:2 in a murine model of lung metastasis reduced the development of lung nodules,
440 whereas C24:1 produced no significant difference compared to the control group. We also
441 discovered that C24:2 induced more cytokine production both *in vitro* and *in vivo* than
442 C24:1, and the profile was more skewed to Th1 cytokines. This skewing toward IFNγ

443 contributed to protection and was supported by the finding that C24:2 treatment was not
444 effective in reducing lung metastases in *Ifng*-KO mice compared to WT mice.
445
446 Our *in vivo* analysis of plasma cytokines showed that IL-12p70 and sCD40L, as well as
447 IFNγ, were significantly elevated in C24:2-injected mice compared to C24:1-injected mice.
448 IL-12 production indicates the involvement of APCs in the differences between C24:1 and
449 C24:2. We analyzed the phenotypes of APCs in mice injected with C24:1 and C24:2 and
450 found that C24:2 induced an increase of cDCs, especially cross-presenting CD8α+CD11b–
451 DCs (cDC1s) among APCs. Moreover, the expression of the co-stimulatory molecule,
452 CD80, on cDC1s was significantly elevated in C24:2-injected mice compared to C24:1-
453 injected mice. These results suggest that C24:2 induces not only the expansion of cDC1s but
454 also the activation of these cells. It is worth noting that C24:2 is best compared to C24:1 as
455 they both require intracellular processing and are also injected at a dose of 30 nmol each, in
456 contrast to KRN7000 that was injected at 500 pmol. Recently, Arora P. *et al.* demonstrated a
457 novel concept regarding the different functions between Th1 cell-biasing (α-C-GalCer) and
458 Th2 cell-biasing (C20:2) KRN7000 analogues [23]. They showed that all KRN7000
459 analogues tested (KRN7000, α-C-GalCer, and C20:2) were mainly presented by cDC1s and
460 that qualitative changes in cDC1s contributed to determine the different cytokine profiles
461 induced by KRN7000 analogues [23]. cDC1s have a critical role in inducing tumor
462 immunity *in vivo* [32, 33]. They can efficiently process and load exogenously acquired
463 antigens on MHC-I molecules and present to CD8+ T cells [40]. Moreover, they are the
464 main source of the Th1-polarizing cytokine IL-12 [41, 42], which is involved in the
465 induction of CD4+ Th1 responses through upregulation of the Tbet transcription factor. In

C24:2-injected mice, the expanded and activated cDC1s might contribute, possibly through IL-12, to the substantial amount of IFNγ production, which in turn exerts the anti-tumor effect observed. However, further studies are needed to elucidate the detailed mechanisms of cDC1s in the tumor immunity in our model.

The potential importance of cDC1 cells led us to ask what the hybridoma response would be like when the sulfatides were presented by DCs rather than by cell-free CD1d monomer coated onto plastic. To our surprise, DC presentation completely reversed the specificity of not only C24:2 but also C24:1 sulfatide. Whereas with the cell-fee presentation, it stimulated the type II NKT cell hybridoma XV19 exclusively and not any of the three different type I hybridoma clones tested (Fig. 2), when presented by BMDCs, it stimulated only the type I NKT cell hybridoma DN32 and no longer stimulated XV19 (Fig. 6-7). This is consistent with the finding of Blomqvist M. *et al*. [27] that C24:1 weakly stimulated the type II NKT cell hybridoma XV19 when presented by RMA-S cells, but very little when presented by BMDCs, especially when compared with lysosulfatide. This result suggested that the DCs processed the sulfatide into a lipid that stimulates type I NKT cells, not type II NKT cells.

Sulfatides differ from the corresponding β-galactosylceramides (βGalCer) by the sulfate moiety and from KRN7000 also by the sphingosine rather than phytosphingosine chain and by double bonds in the acyl chain and its length and by the β-linkage of the sugar. Although such β-linked GalCer structures have not been known to have substantial anti-tumor activity or to stimulate type I NKT cells very strongly, βGalCer C24:2 had not been previously tested. Further, it is known that lysosomes contain the enzyme arylsulfatase A, which can

cleave that sulfate moiety [35, 37]. Our data showed that bafilomycin A1, which inhibits endosomal/lysosomal acidification and processing, and arylsulfatase A blockade by sulfite both markedly inhibited the ability of C24:2 on BMDCs to stimulate DN32, supporting our hypothesis that the stimulation of type I NKT cells with C24:2 loaded on DCs is due to endosomal/lysosomal processing by arylsulfatase A to remove the sulfate moiety. Moreover, βGalCer C24:2 structure could stimulate a type I hybridoma and could protect against the tumor *in vivo.* This finding itself was novel because most βGalCer analogues tested previously did not stimulate type I NKT cells very effectively [43-45], even with a phytosphingosine moiety, and the βGalCer C24:2 analogue differed from KRN7000 not only in the beta linkage of the galactose but also in the sphingosine chain. However, this particular βGalCer analogue had never been synthesized or tested before and seems to have unusual properties worthy of further investigation.

If our hypothesis is true, then βGalCer C24:2, derived from C24:2, would activate type I NKT cells *in vivo*, and the protection against tumors by C24:2 would not take place in the absence of type I NKT cells. As predicted, C24:2 did activate type I NKT cells *in vivo*, and *Traj18*-KO mice failed to protect against the CT26 tumor. This outcome confirms the surprising result that it is not C24:2 that directly protects through skewing the function of type II NKT cells, but rather it is the processed form of this molecule, βGalCer C24:2, that protects by stimulating type I NKT cells.

We further showed that this basic phenomenon of endosomal processing of C24:2 to allow it to stimulate type I NKT cells translated in human NKT cells as well, as human peripheral

512 blood type I NKT cells (staining with PBS57-loaded CD1d tetramer) were stimulated by
513 C24:2 in the presence of human APCs and this was inhibited by bafilomycin A1, an
514 endosomal processing inhibitor. This translation greatly increases the potential for
515 applicability to human cancer immunotherapy.
516
517 In summary, we have demonstrated that modification of the ceramide portion of sulfatides
518 could alter the function of a type II NKT cell agonist and its downstream effects on tumor
519 growth. Notably, we found a highly unanticipated result that C24:2 treatment elicited an
520 anti-tumor effect. The surprise was that the difference was based on antigen processing of
521 the glycolipid rather than a different type II NKT cell activity. We believe this is the first
522 demonstration of DC processing of a glycolipid agonist of NKT cells of either type that
523 reverses its function by altering the specificity for the target cell, in this case from
524 stimulating type II NKT cells to stimulating type I NKT cells, which alters the functional
525 activity of the lipid to a protective one. Although some NKT agonist glycolipids may need
526 loading in lysosomes rather than on the cell surface [21, 25], lysosomal processing of NKT
527 cell lipid antigens is unexpected phenomenon, as antigen processing is known primarily for
528 protein antigens presented by classical MHC molecules to conventional T cells with rare
529 exceptions such as the GalGal analogue of KRN7000 [24]. The fact that the processing is
530 not necessary for loading into the MHC-like molecule CD1d, since both C24:2 and its
531 processed form, βGalCer C24:2, can load into CD1d monomer on plastic, but rather to alter
532 the specificity for the target cell, we believe is also a previously unrecognized role of
533 antigen processing in itself, as conventional antigen processing of proteins is to allow
534 loading onto MHC molecules. This study has shed light on the importance of lysosomal

lipid processing, which would affect the NKT cell type to be activated and subsequent immune responses. We propose that these findings may allow the development of new therapeutic targets and agents for cancer immunotherapy.

MATERIALS AND METHODS

Mice

BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA, USA), BALB/c *Ifng*-deficient (*Ifng*-KO), and B6. arylsulfatase A-deficient (*Arsa*-KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA), and BALB/c CD1-deficient (*Cd1*-KO) mice (deficient in both the *Cd1d1* and *Cd1d2* genes) were provided by Dr. M. Grusby (Harvard University, Boston, MA, USA). BALB/c *Traj18* (T cell receptor alpha joining 18)-deficient (*Traj18*-KO) mice were provided by Dr. H. Watarai (Kanazawa University, Kanazawa, Japan). All mice were bred at the National Cancer Institute (NCI). Female mice > 8 weeks of age were used in experiments, and all mice were maintained in a specific pathogen-free animal facility. All possible efforts were made to minimize animal suffering.

Cell lines

Type I NKT cell hybridoma clone DN32.D3 was kindly provided by Dr. A. Bendelac (University of Chicago, Chicago, IL, USA), and 24.9E and 24.8A were kind gifts from Dr. S. Behar (Harvard University, Boston, MA, USA). Type II NKT cell hybridoma clone XV19 was kindly provided by Dr. S. Cardell (University of Gothenburg, Gothenburg, Sweden). All NKT cell hybridoma clones were cultured in RPMI 1640 supplemented with 10% FCS, L-glutamine, sodium pyruvate, non-essential amino acids, streptomycin, penicillin, and 2-mercaptoethanol (0.05 mM). The CT26 colon carcinoma cell line (an N-nitro-N-methylurethane-induced BALB/c murine colon carcinoma) was provided by Dr. N.

Restifo (NCI, NIH, Bethesda, MD, USA) and maintained in RPMI 1640 supplemented with 10% FCS, L-glutamine, sodium pyruvate, non-essential amino acids, streptomycin, and penicillin.

Reagents

Synthetic lipids (KRN7000, C24:0, C24:1, and βGalCer C24:1) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). C24:2, pC24:0, pC24:1, pC24:2, and βGalCer C24:2 were synthesized in our laboratory at the University of Connecticut, as detailed in the supplementary information. All lipids were dissolved in the vehicle (0.5% polysorbate-20) and diluted in PBS or complete RPMI 1640 medium with 10% FBS. For the hybridoma assay, sulfatide analogues and βGalCer analogues were dissolved in dimethyl sulfoxide (DMSO) (Life Technologies, Carlsbad, CA, USA). The purified anti-CD1D1 antibody (clone 20H2) was purchased from Harlan (Indianapolis, IN, USA). Rat IgG was purchased from Sigma Aldrich (St. Louis, MO, USA). An anti-mouse CD3ε antibody was purchased from BioLegend (clone 145-2C11, San Diego, CA, USA). Mouse CD1D1 monomers were kindly provided from the NIH Tetramer Core Facility (Emory University, Atlanta, GA, USA). Fluorescent protein labeled monoclonal antibodies used for flow cytometry are detailed in the supplementary information. Bafilomycin A1 (Sigma-Aldrich, Burlington, MA, USA) was dissolved in DMSO and added to cell cultures at a final concentration of 50 nM 5 minutes before adding lipid antigens. Sodium sulfite (Sigma-Aldrich, Burlington, MA, USA) was dissolved in PBS and used at the concentrations shown.

***In vivo* lung metastasis assay**

584 Mice were injected with a single cell suspension of 5x10e5 CT26 cells in 200 µl of PBS
585 intravenously (i.v.) via the tail vein. Subsequently, a single dose of the glycolipids was
586 diluted from the stock solutions at the desired concentration in 100 µl of PBS and
587 administered intraperitoneally (i.p.) within 1 h after tumor challenge. KRN7000 was
588 administered at 5 µM, whereas sulfatide and βGalCer analogues were administered at 300
589 µM, as previously reported [15, 46]. Mice were sacrificed 12 days after tumor cell
590 inoculation, and pulmonary metastases of CT26 cells were enumerated as previously
591 described [47].
592
593 **Flow cytometric analysis**
594 The surface-stained cells were analyzed with a FACSymphony flow cytometer (BD
595 Biosciences), and the data were processed using FlowJo software (version 10.5.2, Ashland,
596 OR, USA). The following calculation determined the absolute number of cells in each cell
597 subset: total spleen mononuclear cell (MNC) number x the corresponding cell subset
598 proportion to the total CD45+ cells.
599
600 **Bone marrow-derived dendritic cells (BMDCs)**
601 To obtain BMDCs, bone marrow cells were isolated from BALB/c mice and suspended at
602 the concentration of 2x10e5/ml in complete RPMI 1640 medium with 10% FBS in the
603 presence of 20 ng/mL GM-CSF (Peprotech, Rocky Hill, NJ, USA) for 8 days. Fresh
604 medium supplemented with 20 ng/mL GM-CSF was added on day 3 and refreshed on day 6.
605 On day 8 of culture, cells were harvested, washed in complete medium and suspended at

606 4x10e5/ml in complete medium supplemented with 10 ng/ml of GM-CSF for 24 h before
607 they were pulsed with glycolipids and used for lung MNC or hybridoma stimulation.
608
609 **Plate-bound mCD1d hybridoma stimulation assay**
610 The protocol was modified from our previous report [46]. mCD1d monomer was incubated
611 at the concentration of 8 μg/ml with the indicated concentrations of sulfatide or βGalCer
612 analogues in pH 5.0 sodium acetate buffer containing 10 μg/ml saposin C (ENZO Life
613 Sciences, Farmingdale, NY, USA). After incubation at 37C overnight, the acidic buffer was
614 replaced with PBS and concentrated using Amicon Ultra Centrifugal Filter Units 30K
615 (Millipore, Darmstadt, Germany). Then, 96-well flat-bottom plates were coated overnight
616 with 0.5 μg of mCD1d monomers loaded with graded concentrations of sulfatide or βGalCer
617 analogues, or 8.73 μM of KRN7000, or with 10 μg/ml of anti-mouse CD3ε antibody. Plates
618 were then washed with PBS, and 5x10e4 hybridoma cells in 200 μl of complete medium
619 were plated with Rat IgG or 20H2. Cells were cultured at 37C, 5% CO_2. Supernatants from
620 24-h cultures were collected and used in ELISA assays to measure secreted IL-2 using
621 DuoSet kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's
622 instructions. The samples were analyzed in duplicate or triplicate.
623
624 **Cytokine evaluation assay**
625 For comparison of the cytokine secretion profiles, a single-cell suspension of spleen MNCs
626 from naive animals was cultured in 96-well round-bottom plates (1x10e6 cells/well) in the
627 presence of graded concentrations of glycolipids. Cells were cultured at 37C, 5% CO_2 and
628 supernatants were collected at 72 h to measure cytokine secretion levels using DuoSet kit.

Lung MNCs from naïve animals were cultured in 96-well flat-bottom plates (2x10e5 cells/well) with BMDCs (4x10e4 cells/well) pre-pulsed with graded concentrations of glycolipids for 3 h at 37C. Cells were cultured at 37C, 5% CO_2 and supernatants were collected at 96 h to measure cytokine secretion levels using DuoSet kit. To quantify plasma or serum cytokine level, bead-based multiplex LEGENDplex analysis (Biolegend, San Diego, CA, USA) was used following the manufacturer's instructions.

Intracellular cytokine staining of human NKT cells

PBMCs were isolated using the Ficoll-Paque density gradient method and frozen until they were used. Human NKT cells were taken from a healthy donor and were cultured in RPMI 1640 supplemented with 10% FCS, L-glutamine, sodium pyruvate, non-essential amino acids, streptomycin, penicillin, and 2-mercaptoethanol (0.05 mM). Human PBMCs were plated on a 96-well plate at 1x10e6 cells per well and were stimulated with glycolipid (10ug/ml), cell activation cocktail (Biolegend, San Diego, USA), or bafilomycin A1 for 15 h and subsequently treated with brefeldin A (Invitrogen, Carlsbad, CA) for 1 h. Samples were stained with flow cytometry antibodies anti-CD3 (clone SP43-2), anti-IFNγ (clone B27) (BD Biosciences, Franklin Lakes, NJ), PBS57-loaded CD1d tetramer (NIH Tetramer Core Facility, Atlanta, GA) and LIVE/DEAD Fixable Blue Dead Cell Stain (Invitrogen, Carlsbad, CA).

Statistical Analysis

Data are expressed as mean ± SD for each group. Statistical differences between groups were evaluated by the Mann-Whitney test with Holm-Sidak corrections for multiple

comparisons or two-way ANOVA with Dunnet's multiple comparisons for batch effects using GraphPad Prism software (version 8.1.1, GraphPad Software Inc., La Jolla, CA). *p*-values < 0.05 were considered significant.

Study Approval

Animal experiments were conducted in accordance with the protocol approved by the NCI Animal Care and Use Committee. Human peripheral blood mononuclear cells were obtained from the NIH Division of Transfusion Medicine from healthy donors on an IRB-approved NIH protocol (99-CC-0168). Research blood donors provided written informed consent and blood samples were de-identified prior to distribution (Clinical Trials Number: NCT00001846).

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article in its supplementary materials.

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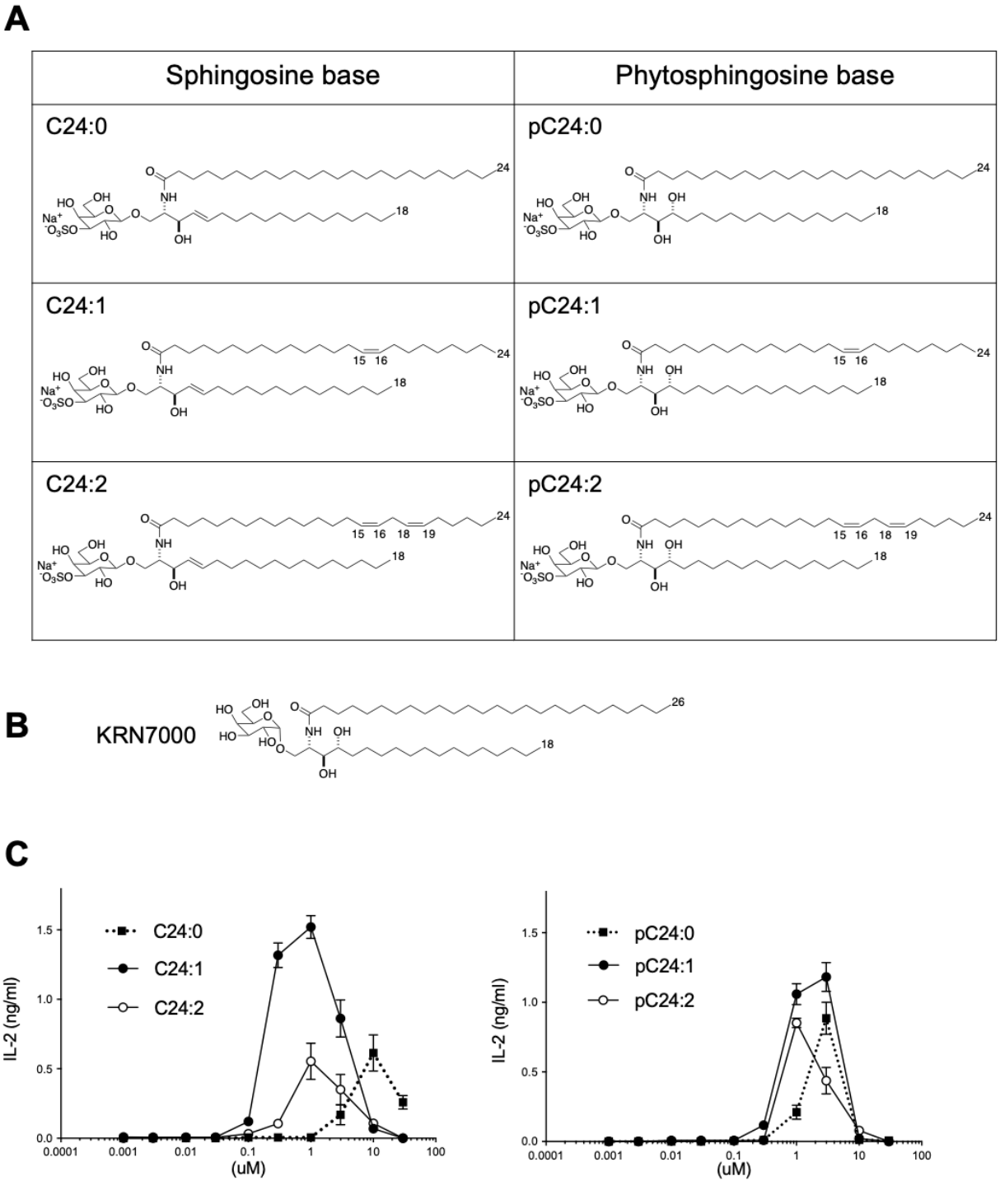
Author contributions: SK, LP, KN, MT, PO, AH, and JB designed the study. KN, LP, KC, JD, SK, AB, SR, KH, and TJ collected the data. KN, LP, SK, JW, JJ, MT, PO, KH, and JB analyzed the data. KN, LP, KC, JD, SC, SR, MT, PO, AH, KH, and JB interpreted the data and discussed the conclusions. KN, LP, KC, JD, and KH contributed equally to this work. The authorship order was determined based on the significance, time, and effort each author invested in the project. KN, LP, MT, PO, AH, KH, and JB wrote the manuscript, which all authors critiqued.

Competing interests: Authors declare that they have no competing interests.

Data and materials availability: Datasets analyzed during the current study are available from the corresponding author on request.

808 **FIGURES**



809

Fig. 1. Sulfatide analogues with sphingosine base or phytosphingosine base stimulate type II NKT cells. (**A**) Structures of sulfatide analogues and (**B**) KRN7000 used in this study.

Analogues C24:2, pC24:0, pC24:1, and pC24:2 are new lipids not previously synthesized or studied as NKT cell agonists to our knowledge. The syntheses of these new compounds (Howell lab) are described in detail in the Supplemental Methods. (**C**) Type II NKT cell hybridoma clone, XV19, was stimulated for 24 h with 0.5 μg of plate-bound CD1d monomers loaded with graded concentrations of sulfatide analogues with sphingosine base (left) or phytosphingosine base (right). IL-2 concentrations in the culture supernatant were measured by ELISA. Data represent two experiments (mean ± SD of triplicate cultures).

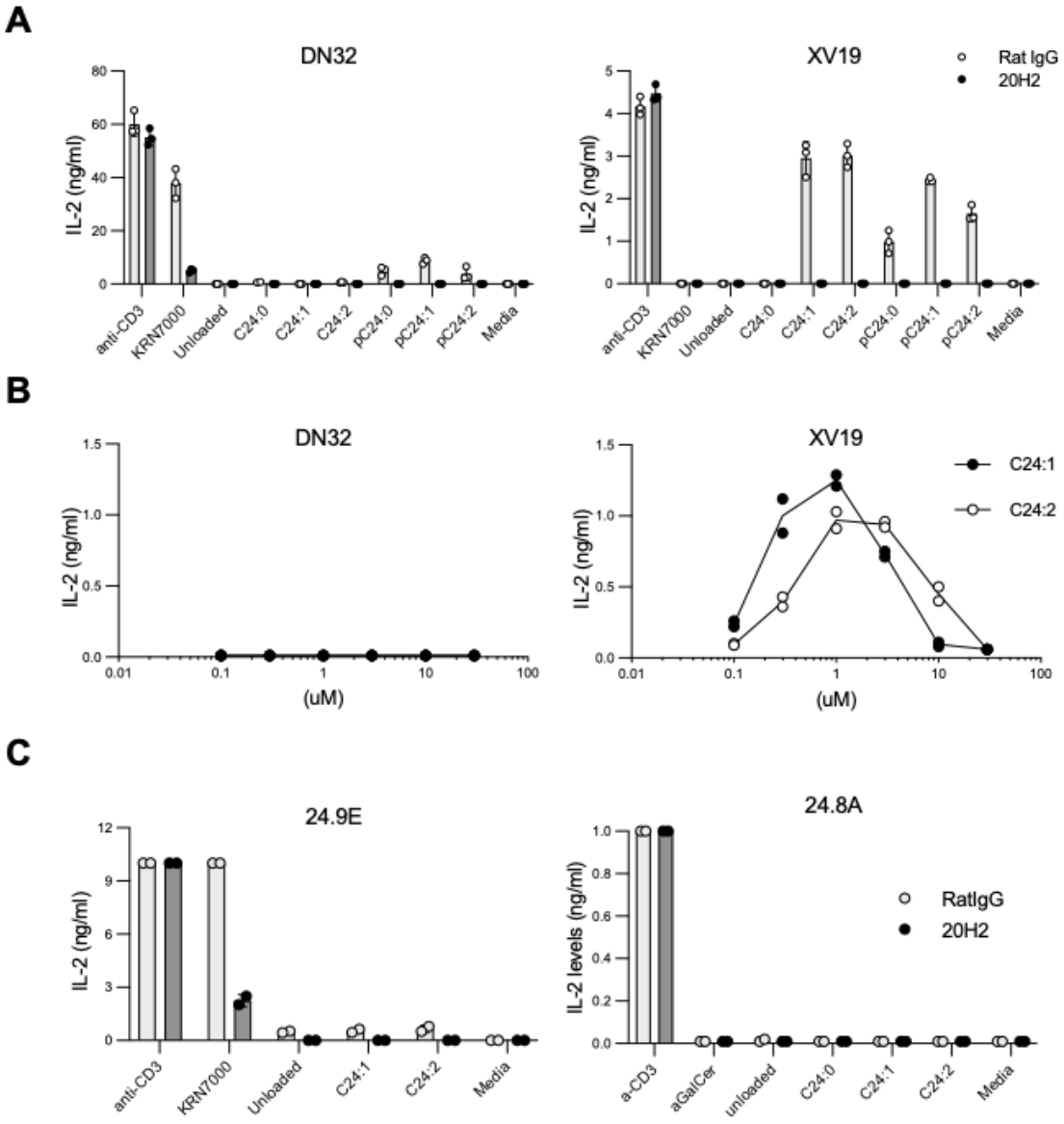


Fig. 2. Sulfatide analogues with sphingosine base stimulate type II NKT hybridoma clone in a CD1d-dependent manner.

Specificities of sulfatide analogues against type I or type II NKT cells are evaluated by analyzing IL-2 concentrations in the culture supernatants of hybridoma clones stimulated with each sulfatide analogues by ELISA. (**A**) Type I NKT hybridoma clone, DN32 (left), and type II NKT hybridoma, XV19 (right), were stimulated 24 h with 0.5 μg of plate-bound

827 mCD1d monomers either unloaded or loaded with each sulfatide analogues (0.5 μM) or
828 KRN7000 (8.73 μM), or with 0.5 μg of plate-bound anti-CD3ε antibody in the presence of
829 10 μg/ml of Rat IgG or anti-CD1d antibody (20H2). (**B**) Type I NKT hybridoma clone,
830 DN32 (left), and type II NKT hybridoma, XV19 (right), were stimulated 24 h with 0.5 μg of
831 plate-bound mCD1d monomers loaded with graded concentrations of C24:1 or C24:2. (**C**)
832 Type I NKT hybridoma clones, 24.9E (left) and 24.8A (right), were stimulated 24 h with 0.5
833 μg of plate-bound mCD1d monomers unloaded or loaded with C24:1, C24:2 (each 1 μM) or
834 KRN7000 (8.73 μM), or with 0.5 μg of plate-bound anti-CD3ε antibody in the presence of
835 10 μg/ml of Rat IgG or anti-CD1d antibody (20H2). Data represent at least two experiments
836 (mean ± SD of triplicate (**A**) or mean ± SD of duplicate (**B** and **C**) cultures).

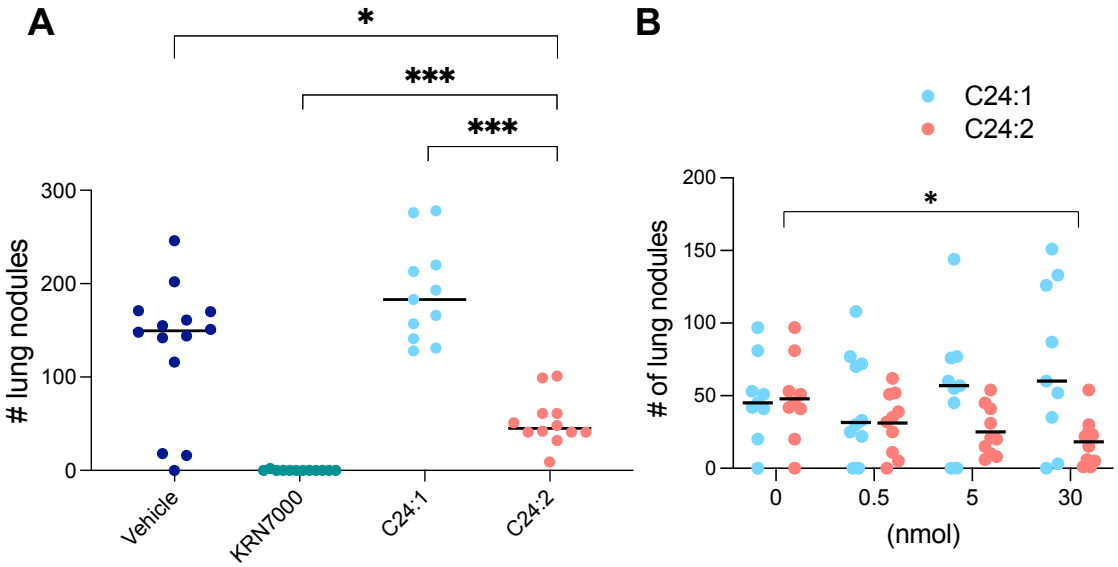


Fig. 3. The effects of sulfatide analogues on tumor immunity.

(**A**) WT mice were injected i.v. via tail vein with 5x10e5 CT26 cells and subsequently injected i.p. with the vehicle used to dissolve the sulfatide analogues, 500 pmol of KRN7000, or 30 nmol of sulfatide analogues. Mice were sacrificed 12 days after tumor challenge, and lung metastases were enumerated. The mean nodule number of each group is expressed in a horizontal bar. Each symbol represents an individual mouse. (**B**) WT mice were injected i.v. into the tail vein with 5x10e5 CT26 cells and subsequently injected i.p. with a graded dose of sulfatide analogues (ranging from 0 to 30 nmol). Mice were sacrificed 14 days after tumor challenge and lung metastases were enumerated (9 to 10 mice per group). Data represent at least two experiments (mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.001$).

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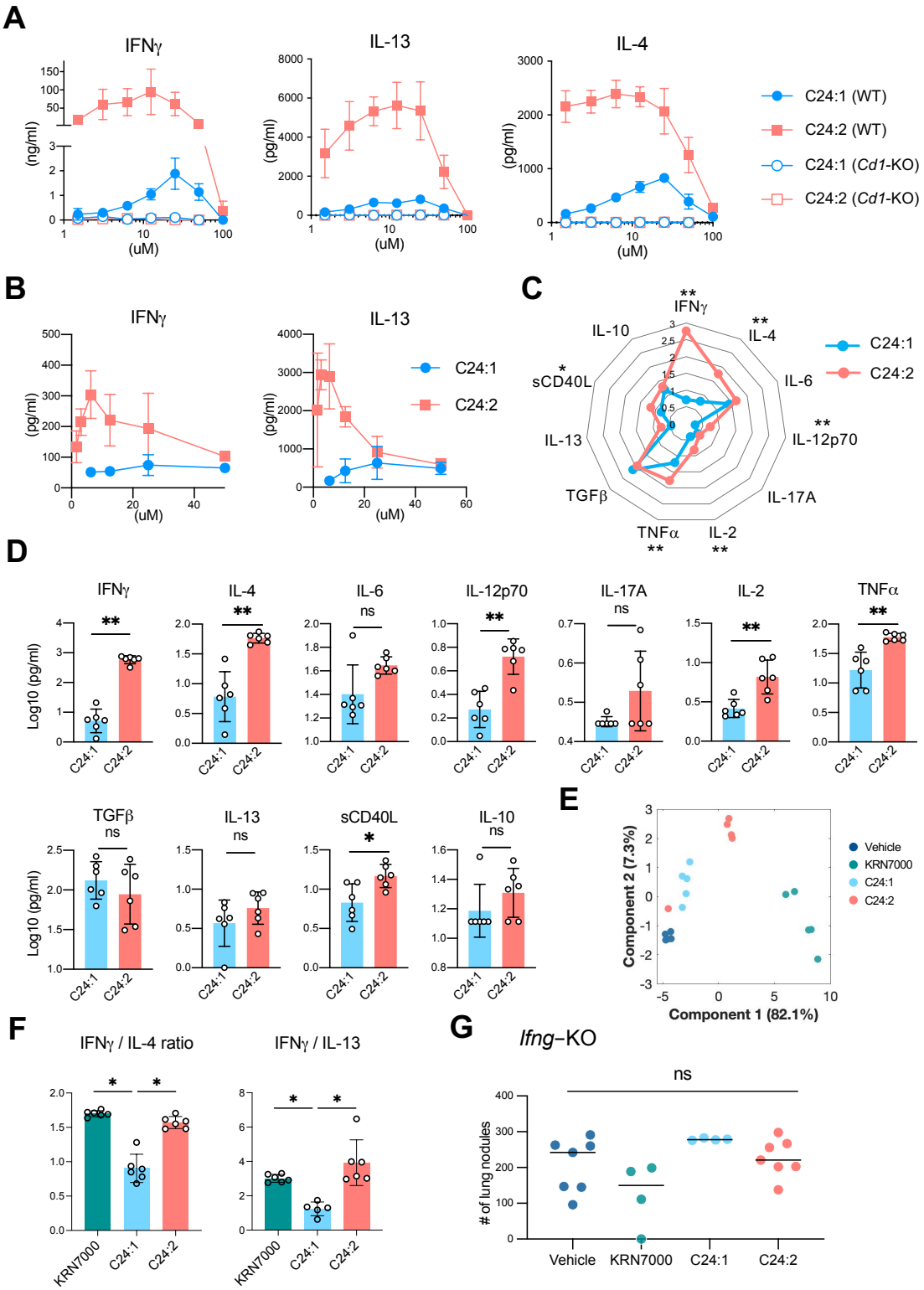

A
IFNγ
IL-13
IL-4
(ng/ml)
(pg/ml)
(uM)
C24:1 (WT)
C24:2 (WT)
C24:1 (Cd1-KO)
C24:2 (Cd1-KO)
B
IFNγ
IL-13
C24:1
C24:2
C
IFNγ
IL-4
IL-6
IL-12p70
IL-17A
IL-2
TNFα
TGFβ
IL-13
sCD40L
IL-10
D
IFNγ
IL-4
IL-6
IL-12p70
IL-17A
IL-2
TNFα
TGFβ
IL-13
sCD40L
IL-10
Log10 (pg/ml)
ns
C24:1
C24:2
E
Component 1 (82.1%)
Component 2 (7.3%)
Vehicle
KRN7000
C24:1
C24:2
F
IFNγ / IL-4 ratio
IFNγ / IL-13
KRN7000
C24:1
C24:2
G
Ifng−KO
# of lung nodules
Vehicle
KRN7000
C24:1
C24:2

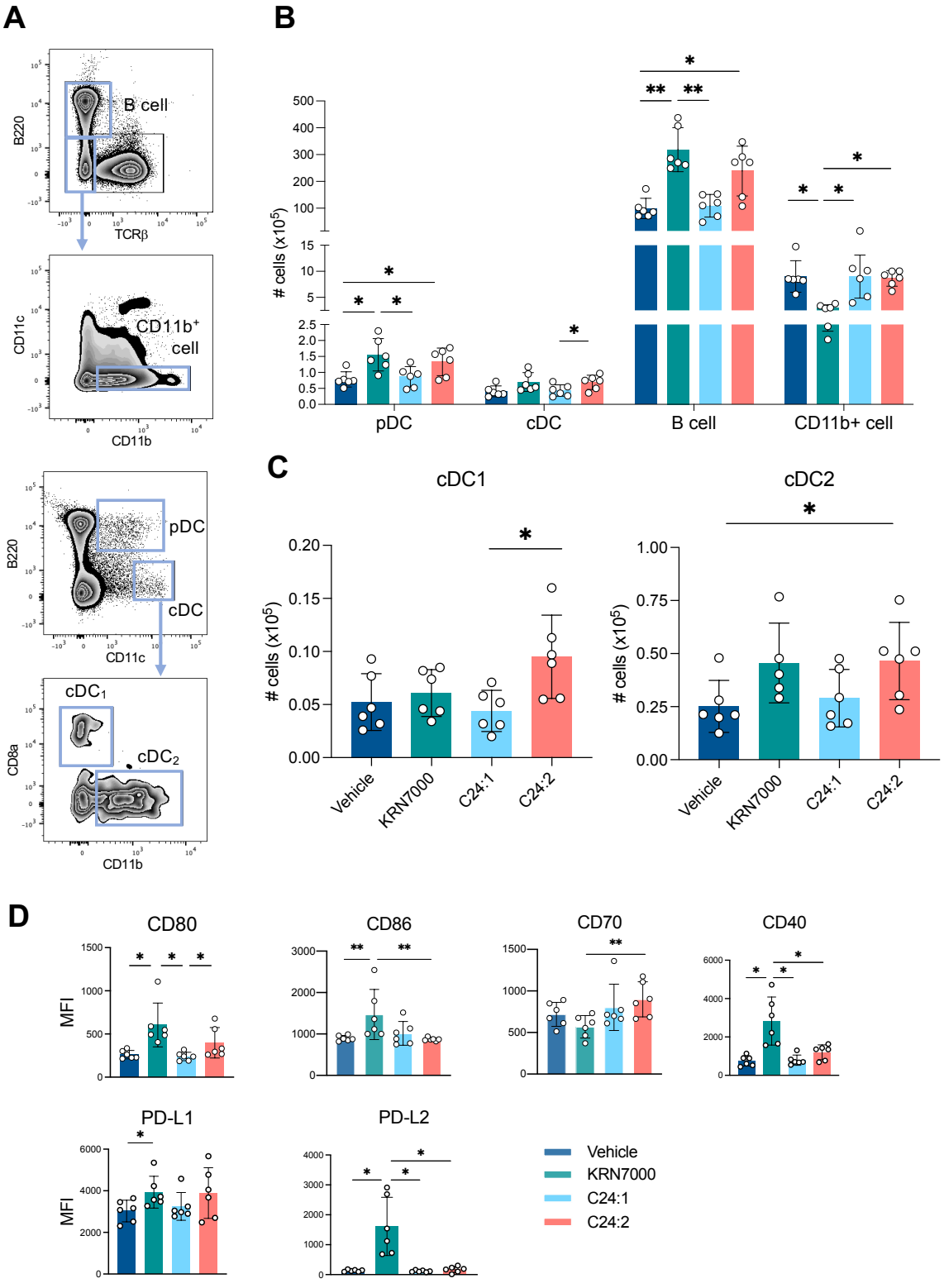
852

853

854 **Fig. 4. Tumor protection induced by C24:2 is IFNγ dependent.**

855 (**A**) *In vitro* cytokine response of spleen MNCs from naive WT mice or *Cd1*-KO mice
856 stimulated 72 h with a graded dose of sulfatide analogues (mean ± SD of triplicate cultures).
857 (**B**) *In vitro* cytokine response of lung MNCs from naive WT mice stimulated 96 h with
858 BMDCs pre-pulsed with a graded dose of sulfatide analogues (mean ± SD of triplicate
859 cultures). (**C** and **D**) A radar plot or bar graph shows the plasma cytokine levels of mice
860 injected i.p. with 30 nmol of sulfatide analogues. Plasma samples were collected 12 h after
861 lipid injection and analyzed. n=6 mice per group. (**E**) Clustering analysis by PCA of serum
862 cytokine profiles of mice injected i.p. with the vehicle used to dissolve the sulfatide
863 analogues, 500 pmol of KRN7000, or 30 nmol of sulfatide analogues is shown. Serum
864 samples were collected 3 h, 6 h, 12 h and 24 h after lipid injection and analyzed. n=5 mice
865 per group. (**F**) The plasma cytokine levels of mice injected i.p. with 500 pmol of KRN7000,
866 30 nmol of C24:1, or 30 nmol of C24:2 are plotted as a ratio between IFNγ and IL-4 (left) or
867 IFNγ and IL-13 (right). Plasma samples were collected 12 h after the lipid injection and
868 analyzed. n=6 mice per group. (**G**) *Ifng*-KO mice were injected i.v. via tail vein with 5x10e5
869 CT26 cells and subsequently injected i.p. with the vehicle used to dissolve the sulfatide
870 analogues, 500 pmol of KRN7000, or 30 nmol of sulfatide analogues. Mice were sacrificed
871 14 days after tumor challenge and lung metastases were enumerated. The mean nodule
872 number of each group is expressed in a horizontal bar. Each symbol represents an individual
873 mouse. Data represent at least two experiments ($^{*}p < 0.05$, $^{**}p < 0.005$, and *ns* not
874 significant across all groups).

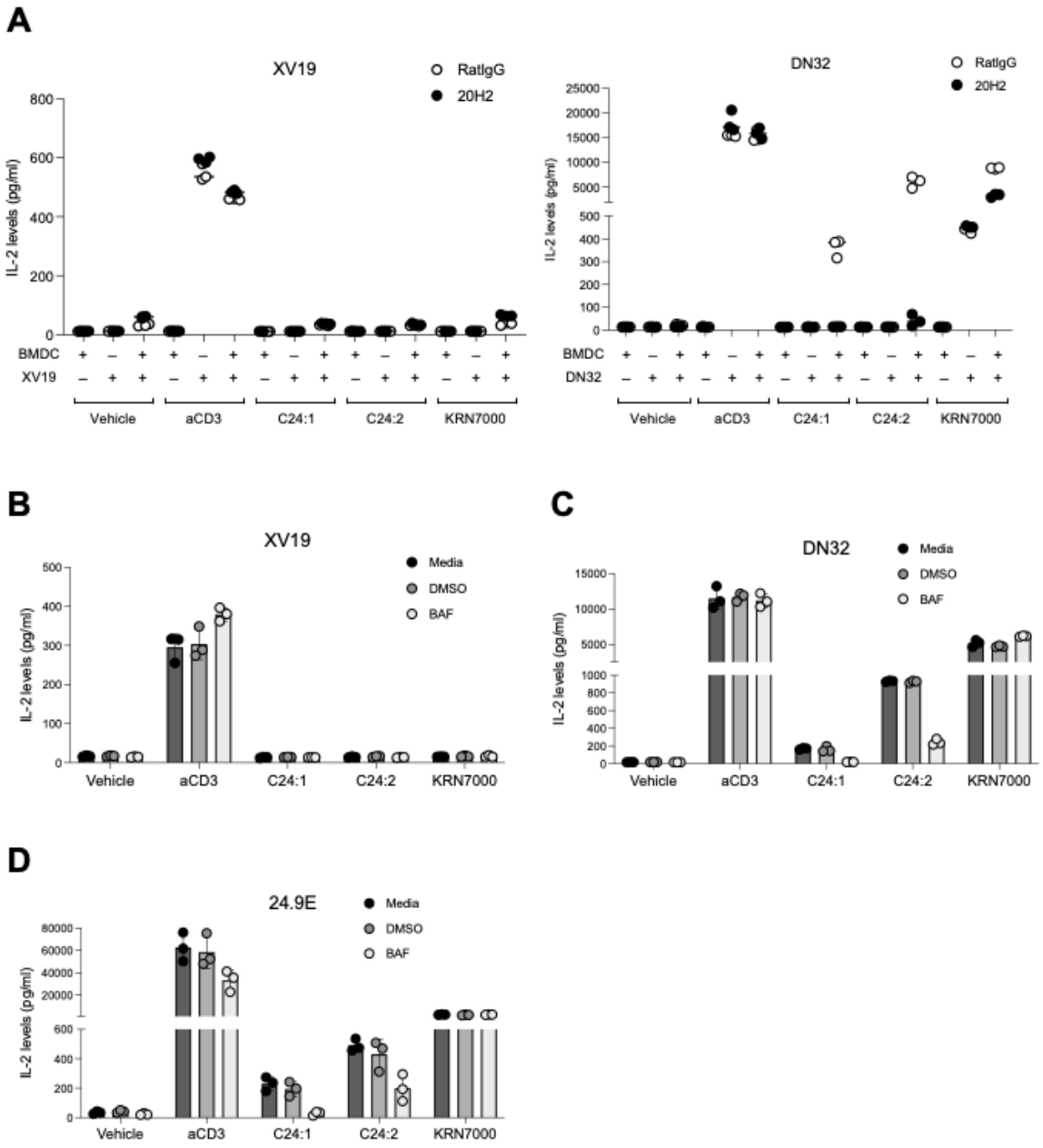
875


A
B220
TCRβ
B cell
CD11c
CD11b
CD11b⁺ cell
B220
CD11c
pDC
cDC
CD8a
CD11b
cDC1
cDC2
B
cells (x10⁵)
pDC
cDC
B cell
CD11b+ cell
C
cDC1
cDC2
cells (x10⁵)
Vehicle
KRN7000
C24:1
C24:2
D
CD80
CD86
CD70
CD40
PD-L1
PD-L2
MFI
Vehicle
KRN7000
C24:1
C24:2

876

Fig. 5. C24:2 induces cDC, especially cDC1, expansion and higher expression of the co-stimulating molecule.

Mice were injected i.p. with the vehicle used to dissolve the sulfatide analogues, 500 pmol of KRN7000 or 30 nmol of sulfatide analogues, and spleens were harvested 24 h later. After staining with mAbs specific for leukocyte markers, flow cytometry was used to gate on each indicated cell type. (**A**) Multiparameter staining for cell-type-specific markers and gating strategy for each population is illustrated by plots. (**B** and **C**) The absolute cell numbers of the indicated cells are shown. (**D**) Splenic cDC1 cells were analyzed by flow cytometry for the indicated cell surface molecules. Data shown are means ± SD. n=6 mice per group. $^{*}p < 0.05$, $^{**}p < 0.005$, *ns* not significant.



888

889 **Fig. 6. Sulfatide analogues stimulate type I NKT cells but lose their ability to stimulate**

890 **type II NKT cells when presented by BMDCs.**

891 The reactivity of type I or type II NKT cells stimulated with BMDCs pre-pulsed with

892 sulfatide analogues was evaluated by analyzing IL-2 levels in the culture media by ELISA.

893 (**A**) Type II NKT hybridoma clone, XV19 (left), and type I NKT hybridoma, DN32 (right),

were stimulated 16 h with BMDCs pre-pulsed with sulfatide analogues (10 μM) or KRN7000 (25 nM), or with 0.25 μg of plate-bound anti-CD3ε antibody in the presence of 10 μg/ml of Rat IgG or anti-CD1d antibody (20H2) (mean ± SD of triplicate cultures). (**B, C,** and **D**) Type II NKT hybridoma clone, XV19 (**B**), and type I NKT hybridoma clones, DN32 (**C**) and 24.9E (**D**), were stimulated 16 h with BMDCs pre-pulsed with sulfatide analogues (10 μM) or KRN7000 (25 nM), or with 0.25 μg of plate-bound anti-CD3ε antibody in the presence of culture media, 0.03% DMSO, or 50 nM bafilomycin A1 (BAF). Data represent two experiments (mean ± SD of triplicate cultures).

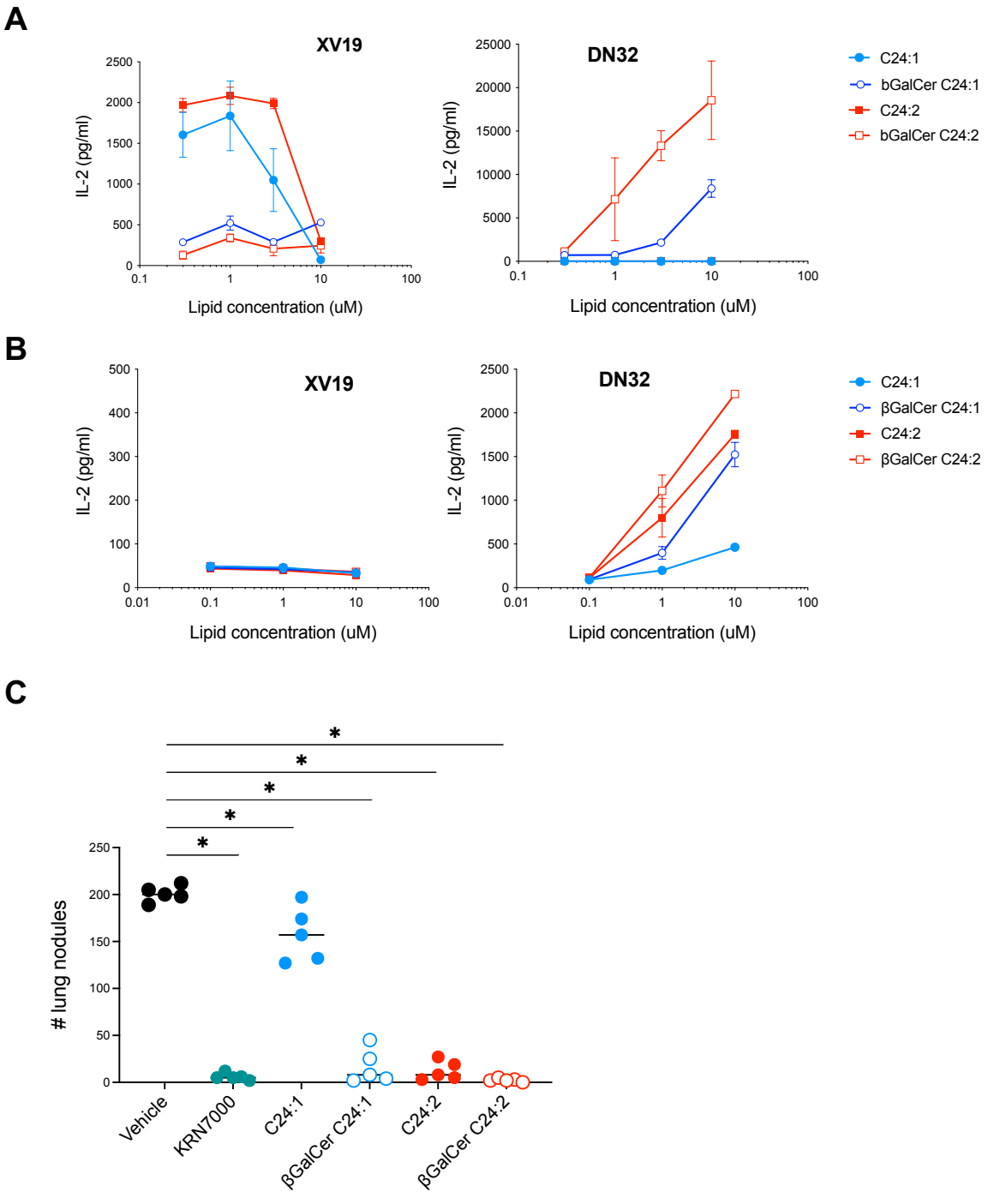
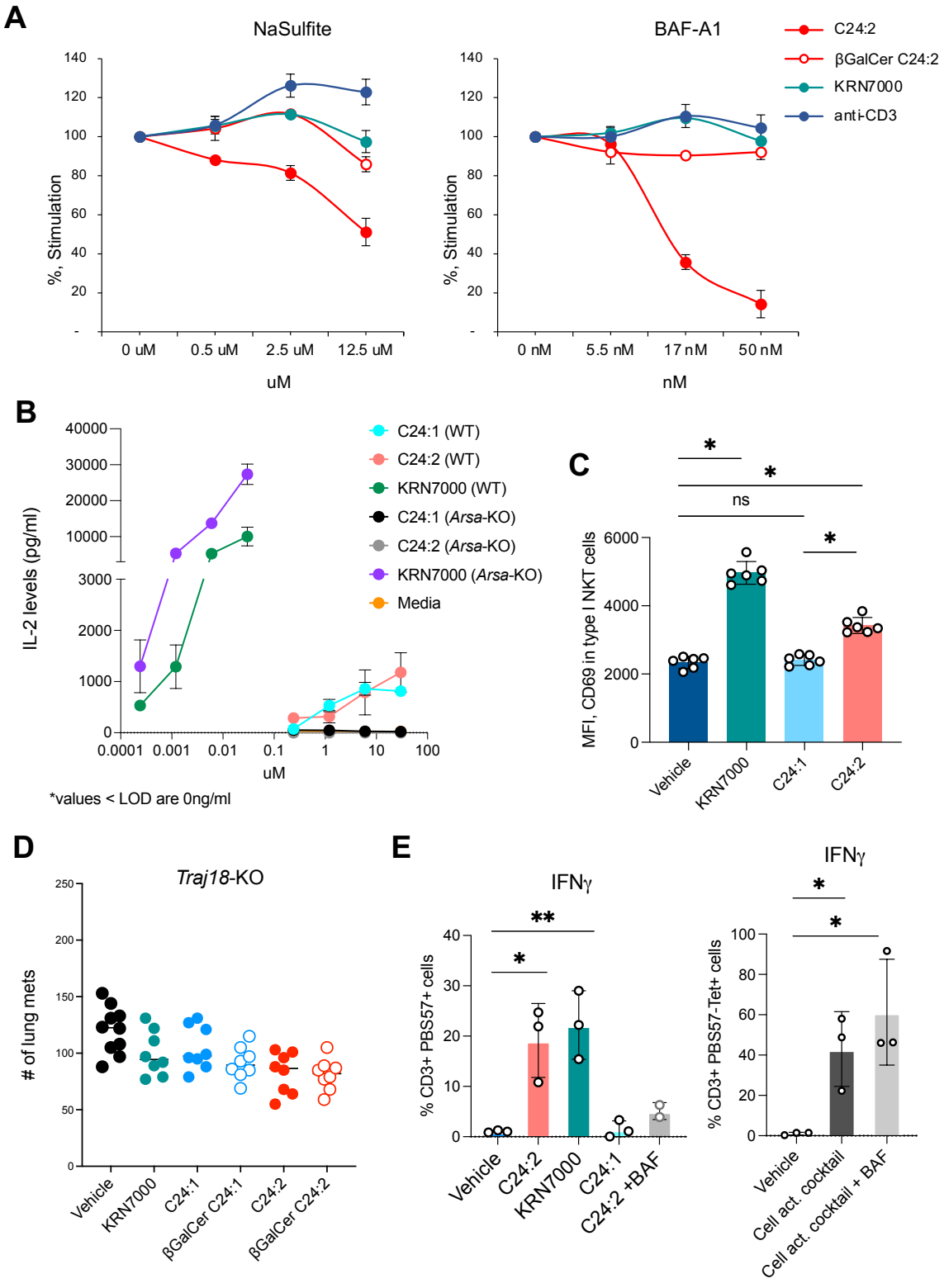

A
XV19
DN32
IL-2 (pg/ml)
Lipid concentration (uM)
C24:1
bGalCer C24:1
C24:2
bGalCer C24:2
B
XV19
DN32
IL-2 (pg/ml)
Lipid concentration (uM)
C24:1
βGalCer C24:1
C24:2
βGalCer C24:2
C
lung nodules
Vehicle
KRN7000
C24:1
βGalCer C24:1
C24:2
βGalCer C24:2

Fig. 7. βGalCer stimulates type I NKT cells more potently than the corresponding sulfatides.

(**A**) Type II NKT hybridoma clone, XV19 (left), and type I NKT hybridoma, DN32 (right), were stimulated 24 h with 0.5 μg of plate-bound mCD1d monomers loaded with graded concentrations of sulfatide C24:1, sulfatide C24:2, βGalCer C24:1, or βGalCer C24:2. (**B**) Type II NKT hybridoma clone, XV19 (left), and type I NKT hybridoma, DN32 (right), were stimulated 16 h with BMDCs pre-pulsed with a graded dose of sulfatide analogues or βGalCer analogues (mean ± SD of duplicate cultures). (**C**) WT mice were injected i.v. via tail vein with 5x10e5 CT26 cells and subsequently injected i.p. with the vehicle used to dissolve the sulfatide or βGalCer analogues, 500 pmol of KRN7000, or 30 nmol of sulfatide or βGalCer analogues. Mice were sacrificed 12 days after tumor challenge, and lung metastases were enumerated. The mean nodule number of each group is expressed in a horizontal bar. Each symbol represents an individual mouse. $^{**}p < 0.005$*; ns,* not significant. Data represent at least two experiments (mean ± SD).

919


A
NaSulfite
BAF-A1
C24:2
βGalCer C24:2
KRN7000
anti-CD3
%, Stimulation
0 uM
0.5 uM
2.5 uM
12.5 uM
uM
0 nM
5.5 nM
17 nM
50 nM
nM
B
C24:1 (WT)
C24:2 (WT)
KRN7000 (WT)
C24:1 (Arsa-KO)
C24:2 (Arsa-KO)
KRN7000 (Arsa-KO)
Media
IL-2 levels (pg/ml)
uM
*values < LOD are 0ng/ml
C
MFI, CD69 in type I NKT cells
ns
Vehicle
KRN7000
C24:1
C24:2
D
Traj18-KO
# of lung mets
Vehicle
KRN7000
C24:1
βGalCer C24:1
C24:2
βGalCer C24:2
E
IFNγ
% CD3+ PBS57+ cells
Vehicle
C24:2
KRN7000
C24:1
C24:2 +BAF
IFNγ
% CD3+ PBS57-Tet+ cells
Vehicle
Cell act. cocktail
Cell act. cocktail + BAF

920

Fig. 8. Inhibition of arylsulfatase A prevents the processing of sulfatides, and their anti-tumor activity is dependent on type I NKT cells.

(**A**) Type I NKT hybridoma clone DN32 cells were stimulated with BMDCs pre-pulsed with either sulfatide C24:2 (10 μM), βGalCer C24:2 (10 μM), KRN7000 (25 nM), or with 0.25 μg of plate-bound anti-CD3 in the presence of titrated 0-12.5 μM of NaSulfide (left) or titrated 0-50 nM of bafilomycin A1 (right) for 16 h. IL-2 level in the culture media was measured by ELISA and %, stimulation of each treatment was calculated relative to the IL-2 level from DN32 cells co-cultured with PBS-treated BMDCs (mean ± SD of duplicate cultures). Data represent at least two experiments (mean ± SD). (**B**) DN32 **c**ells were stimulated for 24 h with 50,000 BMDCs at a 1:1 ratio. *Arsa*-KO BMDCs and WT BMDCs were pre-pulsed with sulfatide analogues, KRN7000, or C24:1. Results are representative data from two experiments (mean ± SD). (**C**) Mice were injected i.p. with the vehicle used to dissolve the sulfatide analogues, 500 pmol of KRN7000 or 30 nmol of sulfatide analogues, and spleens were harvested 12 h later to stain type I NKT cells (CD45+, TCRβ+, PBS57-loaded CD1d-tetramer+) and their activation (CD69, MFI) by flow cytometry. Each symbol represents an individual mouse. $^{**}p < 0.005$*; ns,* not significant. Data represent at least two experiments (mean ± SD). (**D**) *Traj18*-KO mice were injected i.v. via tail vein with 5x10e5 CT26 cells and subsequently injected i.p. with the vehicle used to dissolve the sulfatide or βGalCer analogues, 500 pmol of KRN7000, or 30 nmol of sulfatide or βGalCer analogues. Mice were sacrificed 12 days after tumor challenge, and lung metastases were enumerated. The mean nodule number of each group is expressed in a horizontal bar. Each symbol represents an individual mouse. (**E**) 1x10e6 healthy human PBMCs were cultured with 10 ug/mL of glycolipid (C24:2 with and without BAF 50 nM) for 15 h then 1 h with

944 brefeldin A. Additionally, human PBMCs were cultured with cell activation cocktail in the
945 presence of BAF. (**E**). Quantification of IFNγ+ type I NKT cells after glycolipid treatment.
946 Data represent three experiments pooled (mean ± SD). Results were assessed through two-
947 way ANOVA with Dunnet's multiple comparisons for batch effects. $^{*}p < 0.05$, $^{**}p < 0.01$.