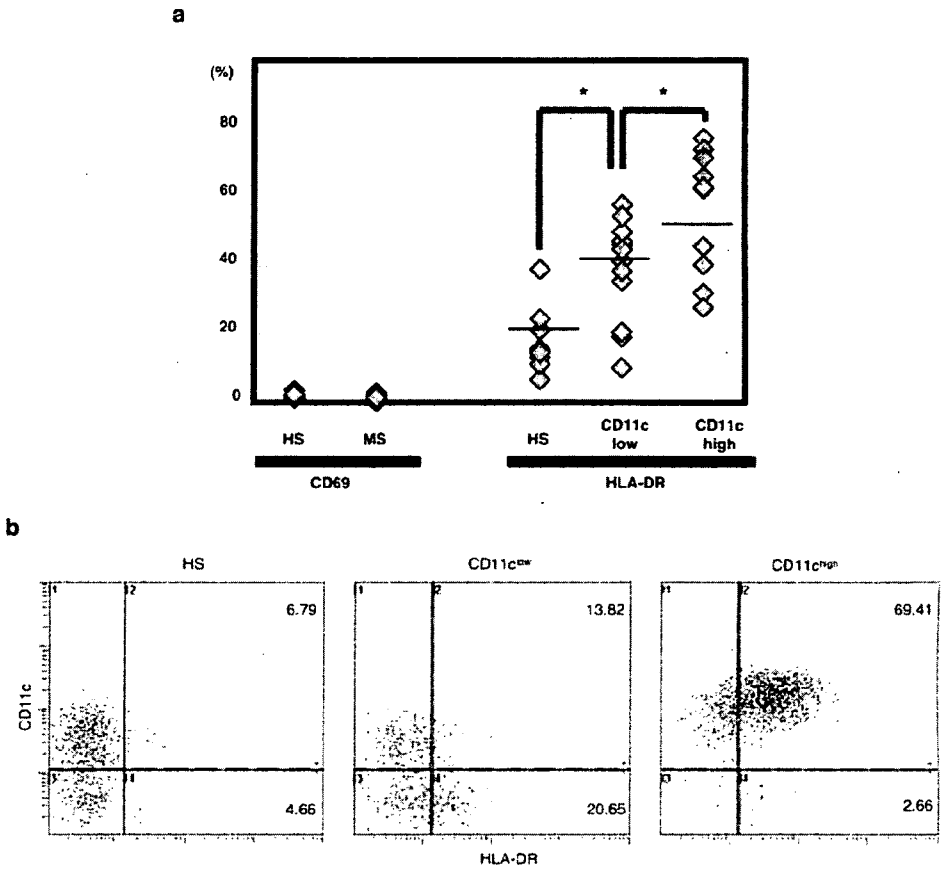

FIGURE 2. Proportions of HLA-DR$^+$ NK cells increase in CD11c^{high} MS. *a*, CD69 and HLA-DR expression on NK cells (CD3$^-$ CD56$^+$ cells). Data are expressed as proportions (percent) of CD69$^+$ cells (7 HS and 16 MS patients in remission) or HLA-DR$^+$ cells (10 HS and 25 MS patients) within whole NK cells. The Student *t* test was used for statistical analysis. Horizontal bars indicate the mean values. *, $p < 0.05$. *b*, Representative expression patterns of HLA-DR vs CD11c on NK cells from a healthy subject (*left*), CD11c^{low} MS (*middle*), and CD11c^{high} MS (*right*).

13 CD11c^{low} and 10 CD11c^{high} MS patients listed in Table II were followed for up to 120 days. In this preliminary exploration, we set the first episode of relapse after blood sampling as an end point. When the neurologist prescribed corticosteroids without knowing any information on the NK cell phenotype, the patient was considered as the dropout at that time point. Remission rate was calculated as Kaplan-Meier survival rate, and statistical difference between CD11c^{low} and CD11c^{high} MS was evaluated with the log-rank test (Fig. 5*a*). At entry, there was no significant difference in the age and disease duration between CD11c^{low} and CD11c^{high} MS (Table II). On analyzing the collected data after completing the study, we found that 8 patients developed a single relapse during the observation period and that the proportion of patients who have had relapse during the follow-up period was greatly higher in CD11c^{high} MS (6 of 10, 60%) than in CD11c^{low} MS (2 of 13, 15.3%). Furthermore, the log-rank test revealed that CD11c^{high} MS relapsed significantly earlier than CD11c^{low} MS ($p = 0.003$), suggesting a possible role of CD11c as a temporal marker for predicting relapse within months after examination. We also explored whether the difference between CD11c^{high} and CD11c^{low} could be influenced by age or sex. When we selected a group of patients younger than 38.5 years old (the mean age of all the patients), a significantly earlier relapse in CD11c^{high} than CD11c^{low} MS was confirmed in this group of patients ($p = 0.0067$, Fig. 5*b*). In the rest of the patients (<38.5 years old), the difference was less clear and not significant ($p = 0.095$). In female patients, CD11c^{high} MS relapsed significantly earlier than CD11c^{low} MS ($p = 0.035$, Fig. 5*c*), whereas this tendency was not statistically significant in male patients ($p = 0.083$). By examining the patients' medical records, we also found that the duration from the last relapse tended to be shorter in CD11c^{high} than CD11c^{low} MS (14.7 ± 12 mo in CD11c^{high} vs 26.7 ± 24.3 mo in CD11c^{low}) and that the mean number of relapses per year was higher in CD11c^{high} MS (0.9 ± 0.6 in CD11c^{high} vs 0.5 ± 0.5 in CD11c^{low}). These are consistent with the postulate that CD11c^{high} MS might be immunologically more active than CD11c^{low} MS (Table II).

Alteration of CD11c expression in the course of MS

We previously described that NK cells may lose NK2 phenotype during relapse (3). It is interesting to know whether the CD11c phenotype also changes in the course of MS. During the follow-up period of 120 days, 8 patients developed a relapse. We were able to take blood samples at relapse before treatment with corticosteroid and then compared the relapse samples with the samples obtained during remission at initiation of the study. As shown in Fig. 6, we saw an obvious tendency that the levels of CD11c expression would decline during relapse ($p < 0.05$). HLA-DR expression on NK cells was also reduced in some patients during relapse, but the difference between remission and relapse samples was not statistically significant.

Expression pattern of CD95 vs CD11c on NK cells in MS

In a previous study, we showed that MS patients could be divided into CD95^{high} and CD95^{low} according to the frequency of CD95$^+$ cells among NK cells (4). Additionally, we examined whether expression of CD11c and CD95 may independently reflect the status of MS. We found no significant correlation between CD95 (%) and CD11c (MFI) on NK cells in MS ($r = 0.29$, $p = 0.16$ with Spearman's correlation coefficient by rank test), indicating that expression of CD95 and CD11c on NK cells may be regulated independently. By setting the upper limits of CD95$^+$ (%) and CD11c MFI as (the average + 2 × SD) of HS (CD95: 44.6%, CD11c: 5.04),

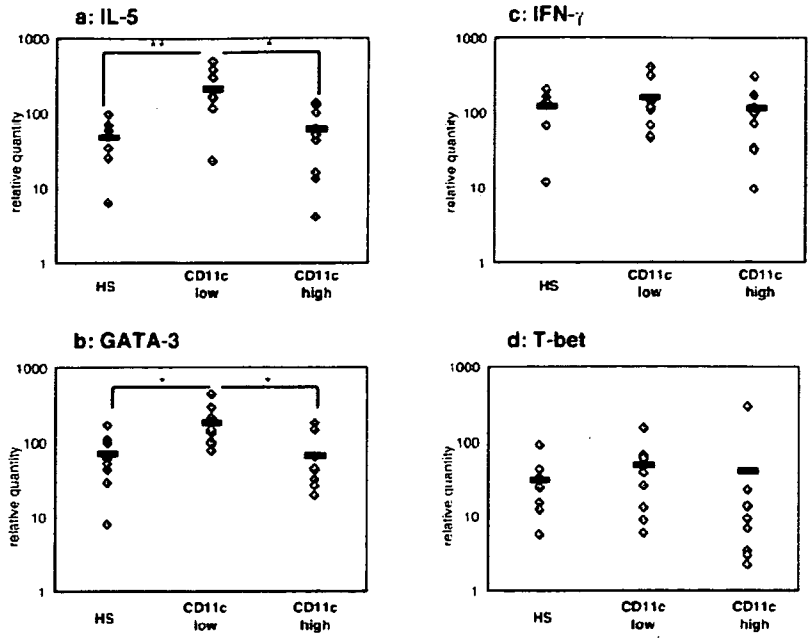

FIGURE 3. IL-5 and GATA-3 mRNA are increased in $CD11c^{low}$ but not in $CD11c^{high}$ MS. Total RNAs were extracted from purified NK cells of HS ($n = 8$), $CD11c^{low}$ ($n = 9$), or $CD11c^{high}$ MS ($n = 8$). mRNA expression of IL-5 (*a*), GATA-3 (*b*), IFN-γ (*c*), and T-bet (*d*) was evaluated by quantitative PCR. The data are normalized to endogenous β-actin expressions in the same samples. ANOVA was used for statistical analysis. Horizontal bars indicate the mean values. *, $p < 0.05$; **, $p < 0.01$.

we then examined whether there is a correlation between CD11c CD95 phenotype and clinical conditions (Fig. 7). Naturally, all the healthy subjects were plotted in the *left lower quadrant* ($CD95^{low}CD11c^{low}$). In contrast, MS patients were plotted in all the four quadrants with differential proportions of patients who have no relapse during 120 days: $CD95^{low}CD11c^{low}$: 3/3 (100%), $CD95^{low}CD11c^{high}$: 1/2 (50%), $CD95^{high}CD11c^{low}$: 8/10 (80%), $CD95^{high}CD11c^{high}$: 2/7 (28.6%). Although the data for $CD95^{low}$ subjects (*lower left* and *lower right*) need to be omitted due to the limited sample size, we found that the difference between $CD95^{high}CD11c^{low}$ and $CD95^{high}CD11c^{high}$ in remission rate was significant with log-rank test ($p = 0.028$). Provided that $CD95^{high}$ patients possessed an increased frequency of memory autoreactive T cells (4), this result is consistent with the idea that when comparable numbers of autoimmune T cells are present in the peripheral circulation, remission of MS is more stable in patients with $CD11c^{low}$ NK cells.

Discussion

Blood examination of systemic autoimmune diseases such as systemic lupus erythematosus usually exhibits measurable abnormalities such as elevation of autoantibodies, which is useful for evaluating activity of disease. In contrast, patients with MS do not accompany such systemic abnormalities in laboratory tests except

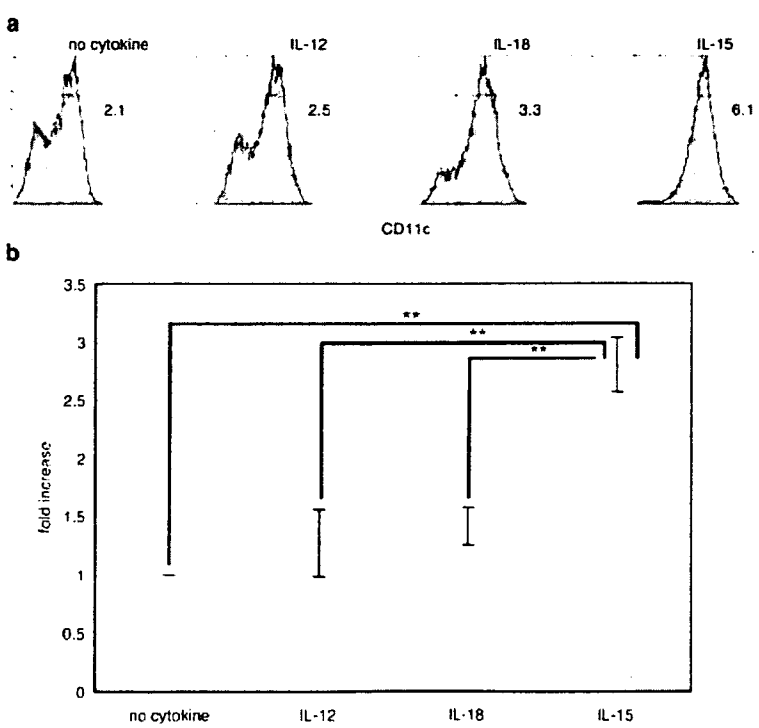

FIGURE 4. CD11c expression on NK cells is upregulated with addition of IL-15. *a*, Purified NK cells were cultured in the absence or presence of IL-12, IL-18, or IL-15. Three days later, the cells were stained with anti-CD11c-PE, -CD3-ECD, and -CD56-PC5 mAb. CD11c expression on NK cells ($CD3^-CD56^+$ cells) is demonstrated as single histogram. Values indicate CD11c MFI of $CD11c^+$ fractions. A representative of three independent experiments is shown. *b*, Data are expressed as mean fold increase of CD11c MFI (the MFI in the presence of cytokine/the MFI in the absence of cytokine) ± SD from three independent experiments. ANOVA was used for statistical analysis. **, $p < 0.01$.

Table I. *Effect of several cytokines on CD11c expression on NK cells*

	No Cytokine	IL-12	IL-18	IL-15	IL-12 + IL-18	IL-4	TNF	GM-CSF	IL-23	IL-8
Expt. 1	1.00[a]	1.19	1.57	**2.90**	ND	ND	ND	ND	ND	ND
Expt. 2	1.00	1.04	1.43	**2.96**	**2.86**	ND	ND	ND	ND	ND
Expt. 3	1.00	1.59	1.25	**2.53**	**3.44**	ND	ND	ND	ND	ND
Expt. 4	1.00	ND	ND	**2.62**	ND	1.19	1.10	0.95	1.14	ND
Expt. 5	1.00	ND	ND	**2.81**	ND	1.24	ND	1.05	1.05	1.00
Mean	1.00	1.27	1.42	**2.77**	**3.15**	1.21	1.10	1.00	1.10	1.00
SD	0.00	0.29	0.16	0.19	0.41	0.03		0.07	0.07	

[a] Purified NK cells were stimulated with cytokines. Data are expressed as fold increase of CD11c MFI (the MFI in the presence of the indicated cytokines/the MFI in the absence of cytokines) in the presence of indicated cytokines. More than a 2-fold increase is highlighted (bold).

in unusual cases. It is currently recognized that autoreactive T cells might be activated and expanded to various degrees in the peripheral blood and peripheral lymphoid organs of MS even during remission (1–4). In fact, our previous work suggests that a higher number of memory autoreactive T cells is linked with unstable disease course (4). If we are able to accurately evaluate the immune status of each patient with a relatively simple test, it should be most helpful in treatment and management of MS. In this line, it is currently of particular importance to identify measurable indicators which would serve as clinically appropriate biomarkers in MS (2).

This study has clarified for the first time to our knowledge that CD11c expression on peripheral NK cells is significantly up-regulated in a major proportion of patients with MS in remission. To obtain insights into the mechanism and the biological meaning of the NK cell expression of CD11c in autoimmune disease MS, we have attempted to clarify the difference between $CD11c^{high}$ and $CD11c^{low}$ patients regarding phenotypes of NK cells, cytokine profile, and temporal clinical activity. We also explored which inflammatory cytokines might induce CD11c on NK cells. According to the NK cell expression of CD11c, we have classified the patients with MS in remission into $CD11c^{high}$ and $CD11c^{low}$. Most notably, NK2 phenotype characterized by predominant IL-5 production was seen in $CD11c^{low}$ patients, but not in $CD11c^{high}$. Consistently, the $CD11c^{high}$ patients were found to be clinically more active than $CD11c^{low}$ as judged by the remission rate during the 120 days after examination. These results indicate that up-regulation of CD11c on NK cells would reflect the temporal disease activity and therefore could be used to identify patients who are likely to exacerbate within months. It has been reported that $CD11c^+$ NK cells in mice could serve as APCs (6, 7). However, we could not reveal Ag presenting capacity of human $CD11c^+$ NK cells (data not shown).

Regarding the mechanism of CD11c induction on NK cells, we have found that in $CD11c^{high}$ patients, HLA-DR is concomitantly up-regulated with CD11c on NK cells (Fig. 2), which suggests that up-regulation of CD11c may represent an activation-induced change. After exploring the culture condition that may induce CD11c on NK cells, we have found that the addition of IL-15 or combination of IL-12 and IL-18 would increase the expression levels of CD11c on NK cells from healthy individuals. Because increased levels of these proinflammatory cytokines are detected in the blood samples of MS (11–13, 18, 19, 23), it is possible that in

Table II. *Information on the patients whose clinical courses were followed for up to 120 days*

Identification No.	Group	Age (years)	Sex	Disease Period (Years)	Total Number of Relapses	Duration from the Last Relapse (mo)	Mean Numbers of Relapse/Year
1	Low	17	F[a]	9.6	2	24	0.2
2	Low	52	M	12.2	9	3	0.7
3	Low	31	F	6.2	13	7	2.1
4	Low	32	F	3.9	1	34	0.3
5	Low	42	F	2.2	1	8	0.5
6	Low	35	M	20	3	88	0.2
7	Low	37	M	8.5	3	50	0.4
8	Low	35	F	2.4	1	38	0.4
9	Low	26	F	4.8	2	10	0.4
10	Low	26	F	1.5	1	8	0.7
11	Low	41	M	5.5	1	24	0.2
12	Low	64	F	4.5	2	8	0.4
13	Low	42	F	6.3	1	45	0.2
Mean ± SD		36.9 ± 12.0		6.7 ± 5.0	3.1 ± 3.7	26.7 ± 24.3	0.5 ± 0.5
14	High	39	M	4.4	2	22	0.5
15	High	31	F	9.2	11	14	1.2
16	High	46	F	7.4	>20[b]	2	ND
17	High	53	F	2.1	4	5	1.9
18	High	59	F	4.9	2	19	0.4
19	High	27	M	9.3	4	9	0.4
20	High	36	F	2.7	1	19	0.4
21	High	34	F	3.8	2	43	0.5
22	High	60	F	3.4	6	10	1.8
23	High	21	F	1.8	2	4	1.1
		40.6 ± 13.4		4.9 ± 2.8	3.8 ± 3.1	14.7 ± 12.0	0.9 ± 0.6

[a] F, Female; M, male.
[b] This value is eliminated from calculation of the mean.

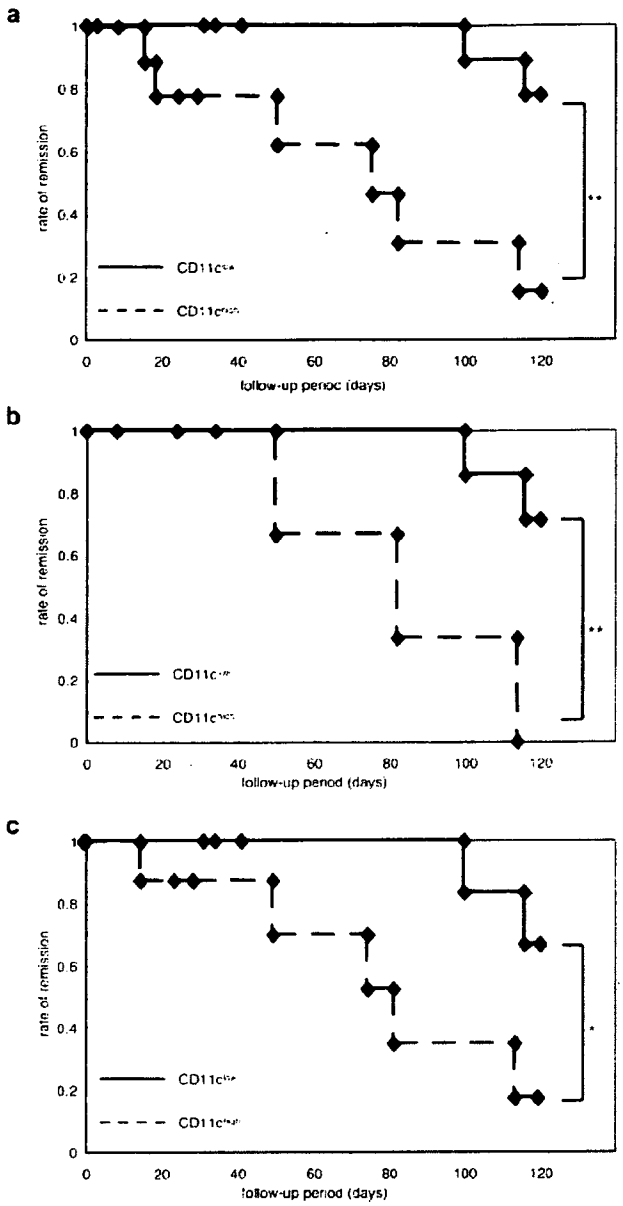

FIGURE 5. Rate of remission is lower in CD11c^{high} MS. The first episode of relapse after blood sampling was set as an end point and clinical course of each patient was followed for up to 120 days. The remission rate was calculated in all (*a*), the younger (*b*), or female (*c*) patients as Kaplan-Meier survival rate, and statistical difference between CD11c^{low} and CD11c^{high} MS was evaluated with log-rank test at day 120. *, $p < 0.05$; **, $p < 0.01$.

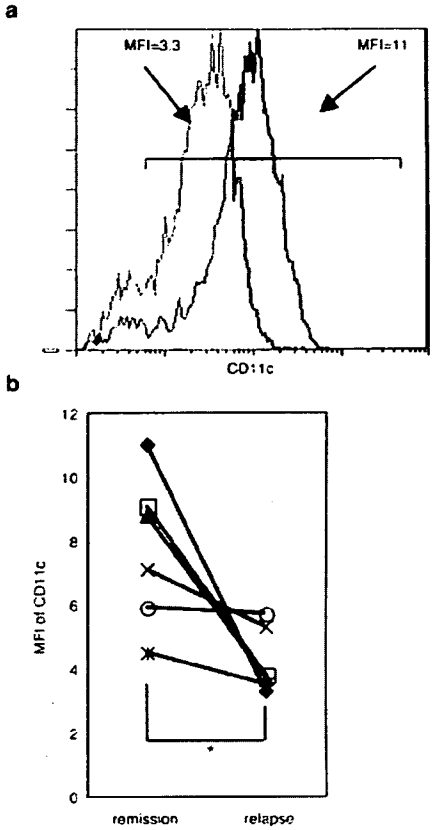

FIGURE 6. Down-regulation of CD11c expression during relapse. *a*, Representative CD11c histograms from the same patient in remission (closed) and relapse (open). Values indicate CD11c MFI of CD11c$^-$ fractions. *b*, Comparison of NK cells from remission and relapse from the same patients ($n = 6$). The data obtained from the same patients are connected with lines. Wilcoxon signed-ranks test was used for statistical analysis. *, $p < 0.05$.

vitro CD11c induction on NK cells may recapitulate the phenotypic alteration of NK cells in CD11c^{high} patients. Interestingly, IL-18 is not only a cytokine able to facilitate IFN-γ production by NK cells in cooperation with IL-12 (25, 26) but is crucial in inducing pathogenic autoimmune responses (21). Furthermore, autoimmune encephalitogenic T cells can induce more serious disease upon adoptive transfer when they are preactivated in the presence of IL-12 and IL-18 (20). Taken together, these results allow us to speculate that the proinflammatory cytokines may be involved in the up-regulation of CD11c on NK cells. Although the relationship between serum cytokine concentration and levels of CD11c expression on NK cells should be estimated in future studies, a previous work (11, 29, 30) showing that a probable link between IL-15 and temporal disease activity, indicates that NK cell expression of CD11c is likely to correlate with the levels of cytokines.

In the Th cell differentiation, specific transcription factors have been identified that play a crucial role in inducing Th1 or Th2 cells. Namely, Th1 differentiation characterized by IFN-γ induction requires a transcription factor T-bet, whereas GATA-3 and c-*maf* act to promote Th2 cytokine production (31–33). Human NK cells cultured in the presence of IL-12 or IL-4 differentiate into NK1 or NK2 populations, reminiscent of Th1 and Th2 cells (5). Whereas NK1 cells produce IL-10 and IFN-γ, NK2 cells would serve as immune regulators by producing IL-5 and IL-13. Notably, up-regulation of GATA-3 has been reported in mouse NK2 cells (17), raising a possibility that Th cells and NK cells might share the same transcription factor for inducing the key cytokine. We have previously reported that IL-5 expression is one of the characteristics of NK cells in the remission state of MS (3). However, it was not excluded that overexpression of IL-5 could be restricted to a proportion of the patients. Here, we have addressed whether NK cells from CD11c^{high} and CD11c^{low} may differ with regard to expression levels of IFN-γ and IL-5 and of their transcription factors T-bet and GATA-3. By measuring the mRNAs, we found that expression levels of IL-5 and GATA-3 are elevated in CD11c^{low} MS but not in CD11c^{high} (Fig. 3). Furthermore, we showed that

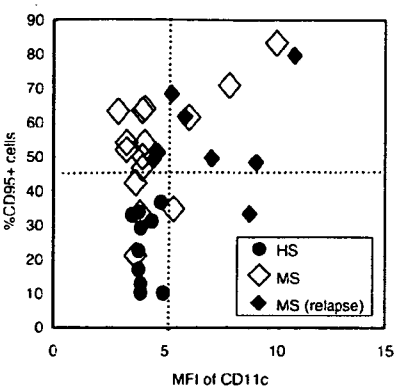

FIGURE 7. Expression pattern of CD95 vs CD11c on NK cells from MS. PBMC from MS or HS were stained with CD95-FITC, CD11c-PE, CD3-ECD, and CD56-PC5. After determining the proportion of $CD95^+$ cells among NK cells and CD11c expression (MFI) of CD11c, we plotted each patient according to the obtained values. Dotted lines represent the upper limits of $CD95^+$ cell (percent) and CD11c MFI for HS as (the average + two times SD) of HS. ●, HS; ◇, MS; ◆, MS patients who relapsed during the 120 days follow-up period.

neither IFN-γ nor T-bet was increased in $CD11c^{high}$ MS. This suggests that NK cells from $CD11c^{low}$ are NK2-biased but those from $CD11c^{high}$ are not, although MS in remission as a whole is NK2-biased as compared with control subjects. More recently, we have observed that stimulation with IL-15 or IL-12 plus IL-18 would decrease IL-5 and GATA-3 mRNA in purified NK cells with reciprocal up-regulation of CD11c (data not shown). This further supports a model that proinflammatory cytokines may play a crucial role in the absence of NK2 bias in $CD11c^{high}$ MS.

To clarify the clinical differences between $CD11c^{high}$ and $CD11c^{low}$, we followed up the clinical course of the patients after blood sampling. Although there was no significant difference in clinical parameters at examination of NK cells, we have found that $CD11c^{high}$ MS showed a significantly earlier relapse than $CD11c^{low}$ MS. This is consistent with our assumption that the absence of NK2 bias in $CD11c^{high}$ MS should imply that regulatory NK cell functions are defective in this group of patients. When we reanalyzed the data regarding various clinical parameters, we found that an earlier relapse in $CD11c^{high}$ than $CD11c^{low}$ MS is more remarkable in the younger group (<38.5 years old) or in female patients. Furthermore, the duration from the last relapse tended to be shorter and the mean number of relapses per year higher in $CD11c^{high}$ MS, supporting that $CD11c^{high}$ MS is more active than $CD11c^{low}$ MS.

When we analyzed expression of CD95 and CD11c on NK cells simultaneously, we found that MS patients in remission could be divided into four subgroups (Fig. 7). When we compared clinical course after examination of NK cell phenotypes, we found that $CD95^{high}CD11c^{high}$ MS relapsed significantly earlier than $CD95^{high}CD11c^{low}$ MS ($p = 0.028$ with log-rank test). This result indicates that $CD95^{high}CD11c^{high}$ MS may be most unstable subgroup of MS, among the patients whose clinical state could be judged as being in clinical remission.

In this study, we have demonstrated that MS patients differentially express CD11c on peripheral blood NK cells and a higher expression of CD11c on NK cells may reflect the temporal disease activity as well as functional alteration of regulatory NK cells. Our results have a clinical implication because of a lack of appropriate biomarker to monitor the immunological status in MS at present. To verify the reliability of this marker, longitudinal examination of CD11c expression on NK cells in the same patients should be performed in the future study.

Disclosures

The authors have no financial conflict of interest.

References

1. Sospedra, M., and R. Martin. 2005. Immunology of multiple sclerosis. *Annu. Rev. Immunol.* 23: 683–747.
2. Bielekova, B., and R. Martin. 2004. Development of biomarkers in multiple sclerosis. *Brain* 127: 1463–1478.
3. Takahashi, K., S. Miyake, T. Kondo, K. Terao, M. Hatakenaka, S. Hashimoto, and T. Yamamura. 2001. Natural killer type 2 bias in remission of multiple sclerosis. *J. Clin. Invest.* 107: R23–R29.
4. Takahashi, K., T. Aranami, M. Endoh, S. Miyake, and T. Yamamura. 2004. The regulatory role of natural killer cells in multiple sclerosis. *Brain* 127: 1917–1927.
5. Peritt, D., S. Robertson, G. Gri, L. Showe, M. Aste-Amezaga, and G. Trinchieri. 1998. Differentiation of human NK cells into NK1 and NK2 subsets. *J. Immunol.* 161: 5821–5824.
6. Homann, D., A. Jahreis, T. Wolfe, A. Hughes, B. Coon, M. J. van Stipdonk, K. R. Prilliman, S. P. Schoenberger, and M. G. von Herrath. 2002. CD40L blockade prevents autoimmune diabetes by induction of bitypic NK/DC regulatory cells. *Immunity* 16: 403–415.
7. Pillarisetty, V. G., S. C. Katz, J. I. Bleier, A. B. Shah, and R. P. Dematteo. 2005. Natural killer dendritic cells have both antigen presenting and lytic function and in response to CpG produce IFN-γ via autocrine IL-12. *J. Immunol.* 174: 2612–2618.
8. Bilsland, C. A., M. S. Diamond, and T. A. Springer. 1994. The leukocyte integrin p150,95 (CD11c/CD18) as a receptor for iC3b: activation by a heterologous β subunit and localization of a ligand recognition site to the I domain. *J. Immunol.* 152: 4582–4589.
9. Stacker, S. A., and T. A. Springer. 1991. Leukocyte integrin P150,95 (CD11c/CD18) functions as an adhesion molecule binding to a counter-receptor on stimulated endothelium. *J. Immunol.* 146: 648–655.
10. Morelli, A. E., A. T. Larregina, W. J. Shufesky, A. F. Zahorchak, A. J. Logar, G. D. Papworth, Z. Wang, S. C. Watkins, L. D. Falo, Jr., and A. W. Thomson. 2003. Internalization of circulating apoptotic cells by splenic marginal zone dendritic cells: dependence on complement receptors and effect on cytokine production. *Blood* 101: 611–620.
11. Blanco-Jerez, C., J. F. Plaza, J. Masjuan, L. M. Orensanz, and J. C. Alvarez-Cermeno. 2002. Increased levels of IL-15 mRNA in relapsing-remitting multiple sclerosis attacks. *J. Neuroimmunol.* 128: 90–94.
12. Huang, W. X., P. Huang, and J. Hillert. 2004. Increased expression of caspase-1 and interleukin-18 in peripheral blood mononuclear cells in patients with multiple sclerosis. *Mult. Scler.* 10: 482–487.
13. Balashov, K. E., D. R. Smith, S. J. Khoury, D. A. Hafler, and H. L. Weiner. 1997. Increased interleukin 12 production in progressive multiple sclerosis: induction by activated $CD4^+$ T cells via CD40 ligand. *Proc. Natl. Acad. Sci. USA* 94: 599–603.
14. Karni, A., D. N. Koldzic, P. Bharanidharan, S. J. Khoury, and H. L. Weiner. 2002. IL-18 is linked to raised IFN-γ in multiple sclerosis and is induced by activated $CD4^+$ T cells via CD40-CD40 ligand interactions. *J. Neuroimmunol.* 125: 134–140.
15. McDonald, W. I., A. Compston, G. Edan, D. Goodkin, H. P. Hartung, F. D. Lublin, H. F. McFarland, D. W. Paty, C. H. Polman, S. C. Reingold, et al. 2001. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. *Ann. Neurol.* 50: 121–127.
16. Lima, M., J. Almeida, M. dos Anjos Teixeira, M. L. Queiros, B. Justica, and A. Orfao. 2002. The "ex vivo" patterns of CD2/CD7, CD57/CD11c, CD38/CD11b, CD45RA/CD45RO, and CD11a/HLA-DR expression identify acute/early and chronic/late NK-cell activation states. *Blood Cells Mol. Dis.* 28: 181–196.
17. Katsumoto, T., M. Kimura, M. Yamashita, H. Hosokawa, K. Hashimoto, A. Hasegawa, M. Omori, T. Miyamoto, M. Taniguchi, and T. Nakayama. 2004. STAT6-dependent differentiation and production of IL-5 and IL-13 in murine NK2 cells. *J. Immunol.* 173: 4967–4975.
18. Nicoletti, F., R. Di Marco, K. Mangano, F. Patti, E. Reggio, A. Nicoletti, K. Bendtzen, and A. Reggio. 2001. Increased serum levels of interleukin-18 in patients with multiple sclerosis. *Neurology* 57: 342–344.
19. Fassbender, K., A. Ragoschke, S. Rossol, A. Schwartz, O. Mielke, A. Paulig, and M. Hennerici. 1998. Increased release of interleukin-12p40 in MS: association with intracerebral inflammation. *Neurology* 51: 753–758.
20. Ito, A., A. Matejuk, C. Hopke, H. Drought, J. Dwyer, A. Zamora, S. Subramanian, A. A. Vandenbark, and H. Offner. 2003. Transfer of severe experimental autoimmune encephalomyelitis by IL-12- and IL-18-potentiated T cells is estrogen sensitive. *J. Immunol.* 170: 4802–4809.
21. Shi, F. D., K. Takeda, S. Akira, N. Sarvetnick, and H. G. Ljunggren. 2000. IL-18 directs autoreactive T cells and promotes autodestruction in the central nervous system via induction of IFN-γ by NK cells. *J. Immunol.* 165: 3099–3104.
22. Takeda, K., H. Tsutsui, T. Yoshimoto, O. Adachi, N. Yoshida, T. Kishimoto, H. Okamura, K. Nakanishi, and S. Akira. 1998. Defective NK cell activity and Th1 response in IL-18-deficient mice. *Immunity* 8: 383–390.

23. Makhlouf, K., H. L. Weiner, and S. J. Khoury. 2001. Increased percentage of IL-12$^+$ monocytes in the blood correlates with the presence of active MRI lesions in MS. *J. Neuroimmunol.* 119: 145–149.
24. Waldmann, T. A. 2004. Targeting the interleukin-15/interleukin-15 receptor system in inflammatory autoimmune diseases. *Arthritis Res. Ther.* 6: 174–177.
25. Fehniger, T. A., M. H. Shah, M. J. Turner, J. B. VanDeusen, S. P. Whitman, M. A. Cooper, K. Suzuki, M. Wechser, F. Goodsaid, and M. A. Caligiuri. 1999. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. *J. Immunol.* 162: 4511–4520.
26. Okamura, H., S. Kashiwamura, H. Tsutsui, T. Yoshimoto, and K. Nakanishi. 1998. Regulation of interferon-γ production by IL-12 and IL-18. *Curr. Opin. Immunol.* 10: 259–264.
27. Young, J. W., P. Szabolcs, and M. A. Moore. 1995. Identification of dendritic cell colony-forming units among normal human CD34$^+$ bone marrow progenitors that are expanded by c-*kit*-ligand and yield pure dendritic cell colonies in the presence of granulocyte/macrophage colony-stimulating factor and tumor necrosis factor α. *J. Exp. Med.* 182: 1111–1119.
28. Detmers, P. A., S. K. Lo, E. Olsen-Egbert, A. Walz, M. Baggiolini, and Z. A. Cohn. 1990. Neutrophil-activating protein 1/interleukin 8 stimulates the binding activity of the leukocyte adhesion receptor CD11b/CD18 on human neutrophils. *J. Exp. Med.* 171: 1155–1162.
29. Kivisakk, P., D. Marusevicius, B. He, M. Soderstrom, S. Fredrikson, and H. Link. 1998. IL-15 mRNA expression is up-regulated in blood and cerebrospinal fluid mononuclear cells in multiple sclerosis (MS). *Clin. Exp. Immunol.* 111: 193–197.
30. Pashenkov, M., M. Mustafa, P. Kivisakk, and H. Link. 1999. Levels of interleukin-15-expressing blood mononuclear cells are elevated in multiple sclerosis. *Scand. J. Immunol.* 50: 302–308.
31. Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100: 655–669.
32. Szabo, S. J., B. M. Sullivan, C. Stemmann, A. R. Satoskar, B. P. Sleckman, and L. H. Glimcher. 2002. Distinct effects of T-bet in TH1 lineage commitment and IFN-γ production in CD4 and CD8 T cells. *Science* 295: 338–342.
33. Zheng, W., and R. A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 89: 587–596.

Stimulation of Host NKT Cells by Synthetic Glycolipid Regulates Acute Graft-versus-Host Disease by Inducing Th2 Polarization of Donor T Cells[1]

Daigo Hashimoto,* Shoji Asakura,* Sachiko Miyake,† Takashi Yamamura,† Luc Van Kaer,‡ Chen Liu,§ Mitsune Tanimoto,* and Takanori Teshima[2]*¶

NKT cells are a unique immunoregulatory T cell population that produces large amounts of cytokines. We have investigated whether stimulation of host NKT cells could modulate acute graft-vs-host disease (GVHD) in mice. Injection of the synthetic NKT cell ligand α-galactosylceramide (α-GalCer) to recipient mice on day 0 following allogeneic bone marrow transplantation promoted Th2 polarization of donor T cells and a dramatic reduction of serum TNF-α, a critical mediator of GVHD. A single injection of α-GalCer to recipient mice significantly reduced morbidity and mortality of GVHD. However, the same treatment was unable to confer protection against GVHD in NKT cell-deficient CD1d knockout ($CD1d^{-/-}$) or $IL\text{-}4^{-/-}$ recipient mice or when $STAT6^{-/-}$ mice were used as donors, indicating the critical role of host NKT cells, host production of IL-4, and Th2 cytokine responses mediated by donor T cells on the protective effects of α-GalCer against GVHD. Thus, stimulation of host NKT cells through administration of NKT ligand can regulate acute GVHD by inducing Th2 polarization of donor T cells via STAT6-dependent mechanisms and might represent a novel strategy for prevention of acute GVHD. *The Journal of Immunology*, 2005, 174: 551–556.

Allogeneic hemopoietic stem cell transplantation (HSCT)[3] cures various hematologic malignant tumors, bone marrow (BM) failures, and congenital metabolic disorders. Emerging evidence suggests that allogeneic HSCT is also useful for treatment of other diseases, including solid tumors and autoimmune diseases (1, 2). However, graft-vs-host disease (GVHD) is a major obstacle that precludes wider application of allogeneic HSCT. The pathophysiology of acute GVHD is complex, involving 1) donor T cell responses to the host alloantigens expressed by host APCs activated by conditioning regimens (i.e., irradiation and/or chemotherapy), and 2) dysregulation of inflammatory cytokine cascades, leading to further T cell expansion and induction of cytotoxic T cell responses (3).

$CD4^+$ helper T cells can be divided into two distinct subpopulations: Th1 and Th2 cells (4). Th1 cells produce IFN-γ and IL-2, whereas Th2 cells produce IL-4, IL-5, and IL-13. Although the role of Th1 and Th2 cytokines in the pathophysiology of acute GVHD is complex and controversial (5–8), Th1 polarization of donor T cells predominantly plays a role in inducing the "cytokine storm" that is seen in several models of acute GVHD (3, 9), whereas Th2 polarization mostly suppresses inflammatory cascades and reduces acute GVHD (10–12). Many properties of dendritic cells (DCs), including the type of signal, the duration of activation, the ratio of DCs to T cells, and the DC subset that presents the Ag, influence the differentiation of naive $CD4^+$ T cells into Th1 or Th2 cells (13). The cytokines that are present during the initiation of the immune responses at the time when the TCR engages with MHC/peptide Ags are critically important for Th cell differentiation (14).

NKT cells are a distinct subset of lymphocytes characterized by expression of surface markers of NK cells together with a TCR. Although the NKT cell population exhibits considerable heterogeneity with regard to phenotypic characteristics and functions (15), the major subset of murine NKT cells expresses a semi-invariant TCR, Vα14-Jα18, in combination with a highly skewed set of Vβs, mainly Vβ8 (16). NKT cells can be activated via their TCR by glycolipid Ags presented by the nonpolymorphic MHC class I-like molecule CD1d expressed by APCs (17). Stimulation of NKT cells rapidly induces secretion of large amounts of IFN-γ and IL-4, thereby influencing the Th1/Th2 balance of conventional $CD4^+$ T cell responses (18). In particular, NKT cells are considered an important early source of IL-4 for the initiation of Th2 responses (19, 20), although these cells are not absolutely required for the induction of Th2 responses (21–23). NKT cells are absent in CD1d knockout ($CD1d^{-/-}$) mice because of defects in their thymic positive selection, which requires CD1d expression on hemopoietic cells, probably double-positive thymocytes (24, 25).

Considering the critical role of cytokines in the development of acute GVHD, we investigated the role of host NKT cells in an experimental model of GVHD, using synthetic NKT cell ligands,

*Biopathological Science, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan; †Department of Immunology, National Institute of Neuroscience, Tokyo, Japan; ‡Department of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN 37232; §Department of Pathology, University of Florida College of Medicine, Gainesville, FL 32610; and ¶Center for Cellular and Molecular Medicine, Kyushu University Hospital, Fukuoka, Japan

Received for publication August 6, 2004. Accepted for publication October 15, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by research funds from the Ministry of Education, Culture, Sports, Science and Technology Grant 15591007 (to T.T.), by the Health and Labor Science Research Grants for Clinical Research for Evidence Based Medicine (to T.T.), by grants from the Ministry of Health, Labour, and Welfare of Japan (to T.Y.), by the Organization for Pharmaceutical Safety and Research (to T.Y.), and by a grant-in-aid for cancer research from the Fukuoka Cancer Society.

[2] Address correspondence and reprint requests to Dr. Takanori Teshima, Center for Cellular and Molecular Medicine, Kyushu University Hospital, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. E-mail address: tteshima@cancer.med.kyushu-u.ac.jp

[3] Abbreviations used in this paper: HSCT, hemopoietic stem cell transplantation; BM, bone marrow; GVHD, graft-vs-host disease; DC, dendritic cell; α-GalCer, α-galactosylceramide; BMT, bone marrow transplantation; TBI, total body irradiation; TCD, T cell depletion; LN, lymph node; WT, wild type.

0022-1767/05/$02.00

α-galactosylceramide (α-GalCer) (26), a glycolipid originally purified from a marine sponge, and its analog, OCH (27). Our findings indicate that stimulation of host NKT cells with NKT ligands can modulate acute GVHD.

Materials and Methods

Mice

Female C57BL/6 (B6, H-2^b) and BALB/c (H-2^d) mice were purchased from Charles River Japan. IL-4$^{-/-}$ B6 and STAT6$^{-/-}$ BALB/c mice were purchased from The Jackson Laboratory. CD1d$^{-/-}$ B6 mice were established by specific deletion of the CD1d1 gene segment (22). Mice, between 8 and 16 wk of age, were maintained in a specific pathogen-free environment and received normal chow and hyperchlorinated drinking water for the first 3 wk post-bone marrow transplantation (BMT). All experiments involving animals were performed under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources, Okayama University Advanced Science Research Center.

Bone marrow transplantation

Mice were transplanted according to a standard protocol described previously (28). Briefly, B6 mice received lethal total body irradiation (TBI; x-ray), split into two doses separated by 6.5 h to minimize gastrointestinal toxicity. Recipient mice were injected with 5 × 10^6 BM cells plus 5 × 10^6 spleen cells from either syngeneic (B6) or allogeneic (BALB/c) donors. T cell depletion (TCD) of donor BM cells was performed using anti-CD90 MicroBeads and the AutoMACS system (Miltenyi Biotec) according to the manufacturer's instructions. Donor cells were resuspended in 0.25 ml of HBSS (Invitrogen Life Technologies) and injected i.v. into recipients on day 0. Survival was monitored daily. The degree of systemic acute GVHD was assessed weekly by a scoring system incorporating five clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity, as described (29).

Glycolipids

α-GalCer, (2S,3S,4R)-1-*O*-(α-D-galactopyranosyl)-2-(*N*-hexacosanoylamino)-1,3,4-octadecanetriol (KRN7000), was synthesized and provided by Kirin Brewery Company (30). A homologue of α-GalCer, OCH, was selected from a panel of synthesized α-GalCer analogues by replacing the sugar moiety and/or truncating the aliphatic chains, because of its ability to stimulate enhanced IL-4 and reduced IFN-γ production by NKT cells, as previously described (27, 31). BMT recipient mice were injected i.p. with α-GalCer or OCH (100 μg/kg) immediately after BMT on day 0. Mice from the control groups received the diluent only.

Flow cytometric analysis

mAbs used were FITC- or PE-conjugated anti-mouse CD4, H-2K^b, and H-2K^d (BD Pharmingen). Cells were preincubated with 2.4G2 mAb (rat anti-mouse FcγR) for 10 min at 4°C to block nonspecific binding of labeled Abs, and then were incubated with the relevant mAbs for 15 min on ice. Finally, cells were washed twice with 0.2% BSA in PBS and fixed. After lysis of RBCs with FACS lysing solution (BD Pharmingen), cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). 7-Amino-actinomycin D (BD Pharmingen)-positive cells (i.e., dead cells) were excluded from the analysis. Fluorochrome-conjugated irrelevant IgG were used as negative controls. At least 5000 live events were acquired for analysis.

Cell cultures

Mesenteric lymph nodes (LNs) and spleens were removed from animals 6 days after BMT and four to six mesenteric LNs or spleens from each experimental group were combined. Numbers of cells were normalized for T cells and were cultured in complete DMEM (Invitrogen Life Technologies) supplemented with 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.02 mM 2-ME, and 10 mM HEPES in wells of a 96-well flat-bottom plate, at a concentration of 5 × 10^4 T cells/well with 1 × 10^5 irradiated (20 Gy) peritoneal cells harvested from naive B6 (allogeneic) animals, or with 5 μg/ml plate-bound anti-CD3ε mAbs (BD Pharmingen) and 2 μg/ml anti-CD28 mAbs (BD Pharmingen). Forty-eight hours after the initiation of culture, supernatants were collected for the measurement of cytokine levels.

ELISA

ELISA was performed according to the manufacturer's protocols (R&D Systems) for measurement of IFN-γ, IL-4, and TNF-α levels, as described previously (32). Samples were obtained from culture supernatant and blood from retro-orbital plexus, diluted appropriately, and run in duplicate. Plates were read at 450 nm using a microplate reader (Bio-Rad). The sensitivity of the assays was 31.25 pg/ml for IFN-γ, 7.6 pg/ml for IL-4, and 23.4 pg/ml for TNF-α.

Histology

Formalin-preserved livers and small and large bowels were embedded in paraffin, cut into 5-μm-thick sections, and stained with H&E for histological examination. Slides were coded without reference to prior treatment and examined in a blinded fashion by a pathologist (C. Liu). A semiquantitative scoring system was used to assess the following abnormalities known to be associated with GVHD, as previously described (33): 0, normal; 0.5, focal and rare; 1.0, focal and mild; 2.0, diffuse and mild; 3.0, diffuse and moderate; and 4.0, diffuse and severe. Scores were added to provide a total score for each specimen. After scoring, the codes were broken and data were compiled. Pathological GVHD scores of intestine are the sum of scores for small bowel and colon.

Statistical analysis

Mann-Whitney *U* test was applied for the analysis of cytokine data and clinical scores. We used the Kaplan-Meier product limit method to obtain survival probability, and the log-rank test was applied for comparing survival curves. Differences in pathological scores between the α-GalCer-treated group and the diluent-treated group were examined by two-way ANOVA. We defined $p < 0.05$ as statistically significant.

Results

Administration of α-GalCer stimulates lethally irradiated mice to produce IFN-γ and IL-4

We first determined whether administration of synthetic NKT ligands such as α-GalCer and OCH can stimulate heavily irradiated mice to produce cytokines. B6 mice were given 13 Gy TBI and were injected i.p. with α-GalCer, OCH, or diluent 2 h after TBI. Six hours later, blood samples were obtained, and serum samples were prepared for measurement of IFN-γ and IL-4. TBI alone or BMT itself did not stimulate diluent-treated mice to produce IFN-γ or IL-4 (Fig. 1). Administration of α-GalCer increased serum levels of IFN-γ and IL-4, even in mice receiving TBI. However, serum levels of IFN-γ were much less in irradiated mice than in unirradiated mice. By contrast, the ability of irradiated mice to produce IL-4 to α-GalCer was maintained for 48 h after irradiation. Serum levels of IFN-γ and IL-4 in response to α-GalCer were not altered when irradiated wild-type (WT) mice were injected with 5 × 10^6 BM cells and 5 × 10^6 spleen cells isolated from allogeneic (BALB/c) donors. Furthermore, these cytokine responses were not observed when α-GalCer was injected into irradiated NKT cell-deficient CD1d$^{-/-}$ mice with or without BMT. These results suggest that host NKT cells that survive for at least 48 h after irradiation, rather than from infused donor cells, are critically involved in the production of these cytokines in response to glycolipids. Irradiation appears to impair the ability of mice to produce IFN-γ while preserving IL-4 production in response to α-GalCer. Similar cytokine profiles were observed when OCH was administered (data not shown).

Administration of α-GalCer to recipients polarizes donor T cells toward Th2 cytokine production after allogeneic BMT

Induction of GVHD fundamentally depends upon donor T cell responses to host alloantigens. We next evaluated the effect of glycolipid administration on donor T cell responses after allogeneic BMT in a well-characterized murine model of acute GVHD (BALB/c→ B6) directed against both MHC and multiple minor histocompatibility Ags. Lethally irradiated B6 mice were transplanted with 5 × 10^6 BM cells and 5 × 10^6 spleen cells from

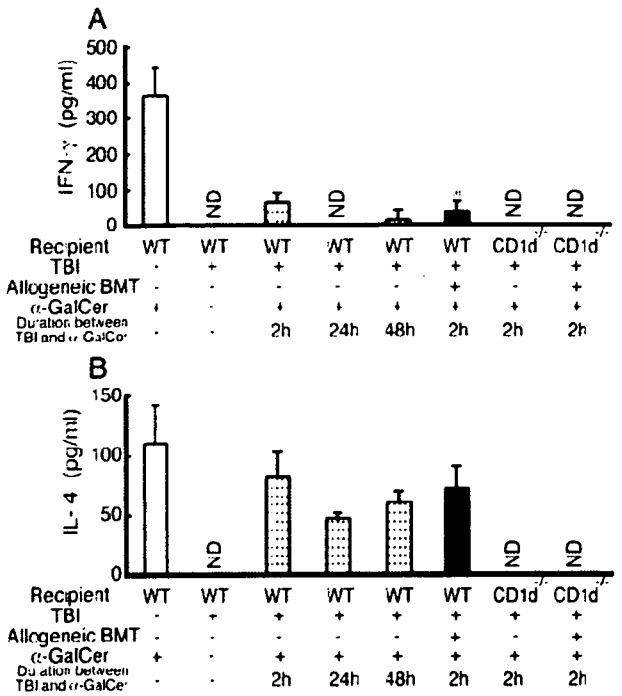

FIGURE 1. Cytokine responses to α-GalCer in lethally irradiated mice with or without BMT. WT and CD1d$^{-/-}$ B6 mice received 13 Gy TBI. Two, 24, or 48 h later, mice were injected i.p. with α-GalCer (100 μg/kg) or diluent. A cohort of animals were transplanted with allogeneic BM cells (5×10^6) and spleen cells (5×10^6) from WT BALB/c donors immediately after TBI, followed by injection of α-GalCer 2 h after TBI. Six hours after the administration of α-GalCer, serum samples were collected, and levels of IFN-γ (*A*) and IL-4 (*B*) were measured. α-GalCer-treated control mice without TBI (□), recipients of TBI plus α-GalCer (▨), and recipients of TBI, allogeneic BMT, and α-GalCer (■) are shown ($n = 3$ per group). Results represent one of three similar experiments and are shown as mean ± SD. *, $p < 0.05$ vs nonirradiated controls. ND, Not detected.

either syngeneic (B6) or allogeneic (BALB/c) donors. Immediately after BMT, B6 recipients were injected i.p. with either α-GalCer or diluent. Six days after BMT, T cells isolated from mesenteric LN of recipient mice were cultured with irradiated B6 peritoneal cells or with anti-CD3ε mAbs and anti-CD28 mAbs for 48 h, and cytokine levels in the supernatant were determined. Flow cytometric analysis showed that >97% of LN T cells from both control recipients and α-GalCer-treated recipients were donor derived, as assessed by H-2^d vs H-2^b expression. T cells from α-GalCer-treated mice secreted significantly less IFN-γ, but more IL-4, in response to host alloantigens (Fig. 2, *A* and *B*) or to CD3 stimulation (Fig. 2, *C* and *D*) compared with those from controls. Similar results were obtained when T cells isolated from spleens were stimulated by anti-CD3ε and anti-CD28 mAbs. T cells from α-GalCer-treated mice secreted significantly less IFN-γ (18 ± 2 vs 164 ± 6 ng/ml), but more IL-4 (1022 ± 114 vs 356 ± 243 pg/ml), compared with controls. These results demonstrate that a single injection of α-GalCer to BMT recipients polarizes donor T cells toward Th2 responses after allogeneic BMT.

In α-GalCer-treated mice, serum levels of IFN-γ were dramatically reduced on day 6 compared with controls (Fig. 3*A*), and IL-4, which is usually hardly detectable in serum in this model, failed to be detected in the serum of mice of either group (data not shown). This impaired Th1 response of donor T cells was associated with a marked reduction of TNF-α levels in α-GalCer-treated mice (Fig. 3*B*).

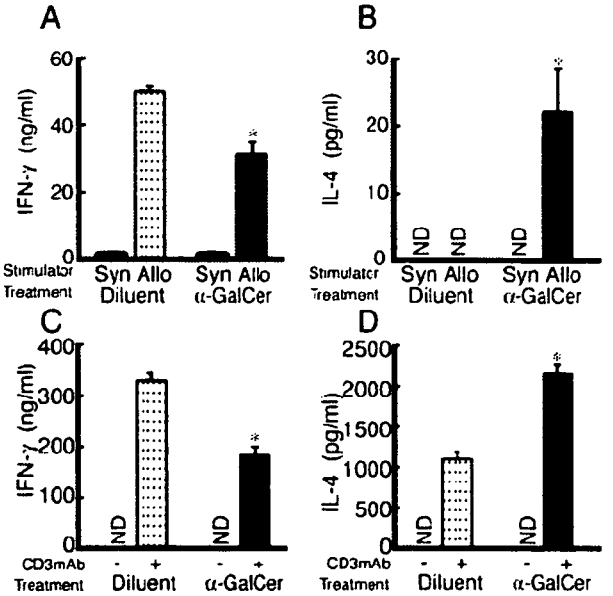

FIGURE 2. Administration of α-GalCer to recipients of allogeneic BMT polarizes donor T cells toward Th2 cytokine secretion. Lethally irradiated (13 Gy) B6 mice were transplanted with BM cells (5×10^6) and spleen cells (5×10^6) isolated from BALB/c mice, followed by injection of either α-GalCer or control diluent. Mesenteric LN cells obtained from diluent-treated recipients (□) and α-GalCer-treated recipients (■) 6 days after BMT were standardized for numbers of CD4$^+$ T cells as 5×10^4/well and were stimulated with 1×10^5/well of allogeneic or syngeneic peritoneal cells (*A* and *B*) or with CD3 (*C* and *D*). After 48 h, cytokine levels in the supernatant were measured by ELISA. Results shown are mean ± SD. *, $p < 0.05$ vs diluent-treated group. ND, Not detected; Syn, syngeneic; Allo, allogeneic.

Administration of α-GalCer or OCH to BMT recipients modulates acute GVHD

We next examined whether immune deviation mediated by administration of glycolipids can modulate acute GVHD. BMT was performed as above and α-GalCer was injected immediately after BMT on day 0. GVHD was severe in allogeneic controls, with 27% survival at day 50. A single injection of α-GalCer significantly improved survival to 86% ($p < 0.05$) (Fig. 4*A*). Allogeneic control mice developed significantly more severe clinical GVHD compared with syngeneic controls, as assessed by clinical GVHD scores (Fig. 4*B*). Clinical GVHD scores were significantly reduced in α-GalCer-treated recipients compared with allogeneic controls, but were greater than in syngeneic controls. Histological analysis showed that administration of α-GalCer significantly suppressed GVHD pathological scores in the intestine ($p < 0.05$). Analysis of donor cell engraftment at day 60 after BMT in spleens showed complete donor engraftment in α-GalCer-treated recipients (>99% H-2K^{d+}/H-2K^{b-} donor chimerism), ruling out rejection or mixed chimerism as a potential cause of GVHD suppression.

Similar protective effects against GVHD were observed in mice treated with OCH, further confirming the protective effects of NKT ligands (Fig. 4*C*). We performed BMT from B6 donors to BALB/c recipients to rule out strain artifacts. Again, a single injection of α-GalCer to BALB/c recipients reduced GVHD and significantly improved survival of animals (Fig. 4*D*).

Host NKT cells and host production of IL-4 are required for suppression of GVHD by α-GalCer

We examined the requirement of host NKT cells in this protective effect of α-GalCer, using NKT cell-deficient CD1d$^{-/-}$ mice as

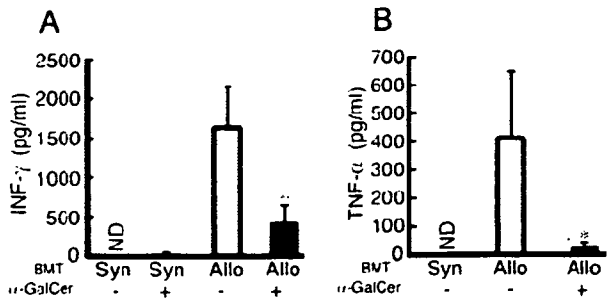

FIGURE 3. A single injection of α-GalCer to recipients of allogeneic BMT markedly reduces serum levels of IFN-γ and TNF-α. WT B6 mice were transplanted as in Fig. 2. Sera (n = 3–10/group) were obtained from diluent-treated (□) and α-GalCer-treated (■) recipients on day 6 after BMT, and serum levels of IFN-γ (*A*) and TNF-α (*B*) were determined. Results from three similar experiments are combined and shown as mean ± SD. *, $p < 0.05$ vs allogeneic, diluent-treated group. ND, Not detected; Syn, syngeneic; Allo, allogeneic.

BMT recipients. Lethally irradiated CD1d$^{-/-}$ mice were transplanted with BM cells and spleen cells from WT BALB/c donors, followed by administration of α-GalCer immediately after BMT on day 0. Protective effects of α-GalCer administration were not observed when CD1d$^{-/-}$ B6 mice were used as recipients, confirming the requirement for host NKT cells (Fig. 5*A*). We next examined the requirement of IL-4 production by host cells in this

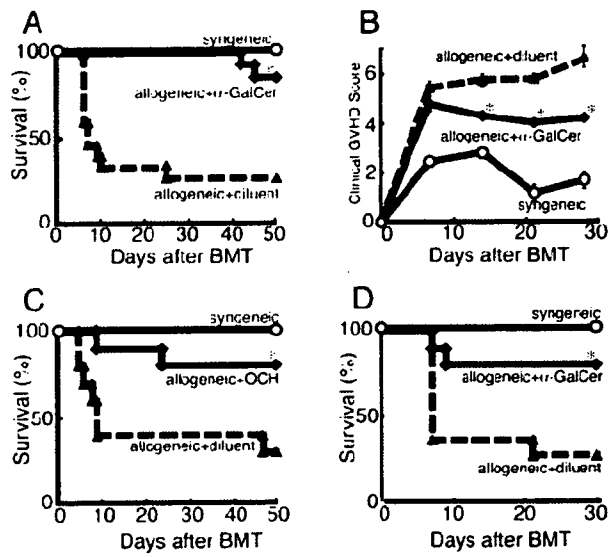

FIGURE 4. A single injection of NKT ligands to BMT recipients modulates acute GVHD. BMT was performed as in Fig. 2. *A*, Survival curves of syngeneic control group (○, solid line; n = 9); allogeneic, diluent-treated recipients (▲, dotted line; n = 15); and allogeneic, α-GalCer-treated recipients (◆, solid line; n = 14) are shown. Data from three similar experiments were combined. *B*, Clinical scores of syngeneic control group (○, solid line); allogeneic, diluent-treated recipients (▲, dotted line); and allogeneic, α-GalCer-treated recipients (◆, solid line) are shown as the mean ± SE. *C*, Survival curves of syngeneic control group (○, solid line; n = 6); allogeneic, diluent-treated recipients (▲, dotted line; n = 10); and allogeneic, OCH-treated recipients (◆, solid line; n = 10) are shown. Data from two similar experiments were combined. *D*, Lethally irradiated (9 Gy) BALB/c mice were transplanted from B6 donors. Survival curves of the syngeneic control group (○, solid line; n = 6); allogeneic, diluent-treated recipients (▲, dotted line; n = 10); and allogeneic, α-GalCer-treated recipients (◆, solid line; n = 10) are shown. Data from two similar experiments were combined. *, $p < 0.05$ vs diluent-treated group.

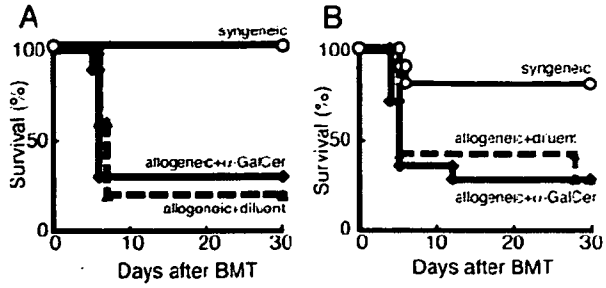

FIGURE 5. Host NKT cells and host IL-4 production are required for suppression of GVHD by α-GalCer. *A*, Lethally irradiated CD1d$^{-/-}$ B6 mice were transplanted as in Fig. 2. Survival curves of syngeneic control group (○, solid line; n = 6); allogeneic, diluent-treated recipients (▲, dotted line; n = 10); and allogeneic, α-GalCer-treated recipients (◆, solid line; n = 10) are shown. Data from two similar experiments were combined. *B*, Lethally irradiated IL-4$^{-/-}$ B6 mice were transplanted as in Fig. 2. Survival curves of syngeneic control group (○, solid line; n = 11); allogeneic, diluent-treated recipients (▲, dotted line; n = 14); and allogeneic, α-GalCer-treated recipients (◆, solid line; n = 14) are shown. Data from three similar experiments were combined.

protective effect. Lethally irradiated IL-4$^{-/-}$ B6 mice were transplanted from WT BALB/c donors and administered α-GalCer as above. α-GalCer did not confer protection against GVHD in IL-4$^{-/-}$ recipients (Fig. 5*B*). Taken together, these results indicate that protective effects of α-GalCer are dependent upon host NKT cells and host production of IL-4.

STAT6 signaling in donor T cells is required for modulation of GVHD by α-GalCer

To determine whether IL-4-induced signaling in donor T cells is critical for the protective effect of glycolipids on GVHD, we used donor spleen cells that lack STAT6 and have impaired IL-4 responses (34, 35). Spleen cells from STAT6$^{-/-}$ BALB/c mice and TCD BM cells from WT BALB/c mice were transplanted after lethal TBI, followed by a single injection of α-GalCer. α-GalCer treatment failed to reduce morbidity and mortality of acute GVHD when STAT6$^{-/-}$ BALB/c donors were used (Fig. 6), demonstrating that STAT6 signaling in donor cells is critical for the protective effect of α-GalCer against GVHD.

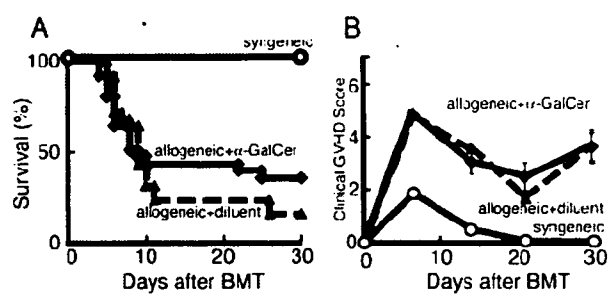

FIGURE 6. The protective effects of α-GalCer against GVHD are dependent upon the STAT6 pathway of donor T cells. Lethally irradiated B6 mice were transplanted with TCD-BM cells (4 × 10^6) from WT BALB/c mice and spleen cells (5 × 10^6) from STAT6$^{-/-}$ BALB/c mice. *A*, Survival curves of the syngeneic control group (○, solid line; n = 15); allogeneic, diluent-treated recipients (▲, dotted line; n = 25); and allogeneic, α-GalCer-treated recipients (◆, solid line; n = 25) are shown. Data from five similar experiments were combined. *B*, Clinical GVHD scores of syngeneic control group (○, solid line); allogeneic, diluent-treated recipients (▲, dotted line); and allogeneic, α-GalCer-treated recipients (◆, solid line) are shown as the mean ± SE.

Discussion

NKT cells are critically involved in the development and suppression of various autoimmune diseases. In experimental models, their regulatory mechanisms mostly depend on IL-4 production and subsequent inhibition of Th1 differentiation of autoreactive $CD4^+$ T cells (18). Previous studies have demonstrated that donor NKT cells regulate acute GVHD in an IL-4-dependent manner when administered together with donor inoculum (36). Considering these immunomodulating functions of NKT cells, we evaluated whether stimulation of host NKT cells could modulate GVHD in a mouse model of this disease.

Administration of α-GalCer stimulates NKT cells to produce both IFN-γ and IL-4 in naive mice, which can promote Th1 and Th2 immunity, respectively (18). We first determined whether administration of synthetic NKT ligands such as α-GalCer and OCH can stimulate heavily irradiated mice to produce cytokines. Surprisingly, irradiation of mice dramatically reduced IFN-γ production in response to α-GalCer, while preserving IL-4 production. This result may account for Th2, but not Th1, polarization of donor T cells by α-GalCer, even in conditions such as allogeneic BMT, which preferentially promotes Th1 polarization. Although mechanisms of selective suppression of IFN-γ production induced by irradiation need to be elucidated, irradiation may modulate the cytokine production profile of NKT cells or neighboring NK cells. Although OCH stimulates NKT cells to predominantly produce IL-4 compared with α-GalCer, resulting in potent Th2 responses (27, 31), both OCH and α-GalCer equally stimulate IL-4 production in irradiated mice and exert equivalent protection against acute GVHD.

Stimulation of host NKT cells by injecting α-GalCer or OCH polarized donor T cells toward Th2 cytokine secretion, resulting in marked reduction of serum IFN-γ levels after BMT. Th2 cytokine responses subsequently inhibited inflammatory cytokine cascades and reduced morbidity and mortality of acute GVHD, as previously described (10–12). Inflammatory cytokines have been shown to be important effector molecules of acute GVHD (37). α-GalCer treatment failed to confer protection against acute GVHD when $STAT6^{-/-}$ BALB/c donors were used, demonstrating that Th2 polarization via STAT6 signaling is critical for this protective effect of α-GalCer, although STAT6-independent Th2 induction has been reported (38, 39).

α-GalCer did not confer protection against GVHD in $CD1d^{-/-}$ or $IL\text{-}4^{-/-}$ recipients. Therefore, the protective effect of α-GalCer against GVHD is dependent upon host NKT cells and host production of IL-4. Sublethal total lymphoid irradiation enriches NKT cells in host lymphoid tissues, and these NKT cells induce Th2 polarization of conventional T cells by IL-4 production, resulting in reduced GVHD (40–42). These findings are consistent with our observation that IL-4 production is critical for the protective effects of NKT cells against acute GVHD. It should be noted, however, that systemic administration of IL-4 is either ineffective or toxic (6). Because the cytokine environment during the initial interaction between naive T cells and APCs is critically important for induction of Th1 or Th2 differentiation (14), local IL-4 production in the secondary lymphoid organs where donor T cells encounter host APCs might be necessary to cause effective Th1→Th2 immune deviation after allogeneic HSCT (43).

Current strategies for prophylaxis and treatment of GVHD primarily target depletion or suppression of donor T cells. These interventions suppress donor T cell activation and are associated with increased risk of infection and relapses of malignant diseases. Th1→Th2 deviation of donor T cells represents a promising strategy to reduce acute GVHD while preserving cytolytic cellular effector functions against tumors and infectious agents (33, 44–47). To achieve Th1→Th2 immune deviation of donor T cells, cytokines have been administered to either donors or recipients in animal models of GVHD. Donor treatment with cytokines such as IL-18 and G-CSF, and recipient treatment with IL-11, induces Th2 polarization of donor T cells and reduces acute GVHD (33, 44, 48). The present study reveals an alternative strategy to induce Th2 polarization of donor T cells by injecting NKT ligands into recipients to activate recipient NKT cells.

Prior studies (36, 40–42, 49) and the current study suggest that both donor and host NKT cells can regulate acute GVHD through their unique properties to secrete large amounts of cytokines and subsequent modulation of adaptive immunity. These studies reveal that there are several ways by which the NKT cell system can be exploited to suppress GVHD. First, administration of donor NKT cells expanded in vitro by repeated stimulation with glycolipid (50) can suppress GVHD (36). Second, total lymphoid irradiation enriches host NKT cells in lymphoid organs and thereby skews donor T cells toward Th2 cytokine production (40–42). Third, as shown here, administration of glycolipid to recipients stimulates host NKT cells to suppress GVHD. A recent phase I trial for patients with various solid tumors demonstrated that administration of α-GalCer was well tolerated with minimal side effects, which included temporal fever, headache, vomiting, chills, and malaise (51). Therefore, α-GalCer treatment may provide an effective and relatively safe option for preventing GVHD.

Cells belonging to the innate arm of the immune system, such as monocytes/macrophages, NKT cells, and NK cells, can produce large amounts of cytokines quickly upon stimulation. Innate immunity can thereby augment donor T cell responses to alloantigens in allogeneic HSCT (3). Our findings reveal a novel role for host NKT cells in regulating GVHD and indicate that stimulation of host innate immunity may serve as an effective adjunct to clinical regimens of GVHD prophylaxis.

Acknowledgments

We thank Kirin Brewery Company for providing synthetic α-GalCer and Keitaro Matsuo for statistical analysis.

References

1. Childs, R., A. Chernoff, N. Contentin, E. Bahceci, D. Schrump, S. Leitman, E. J. Read, J. Tisdale, C. Dunbar, W. M. Linehan, N. S. Young, and A. J. Barrett. 2000. Regression of metastatic renal-cell carcinoma after nonmyeloablative allogeneic peripheral-blood stem-cell transplantation. *N. Engl. J. Med. 343:750.*
2. Burt, R. K., A. E. Traynor, R. Craig, and A. M. Marmont. 2003. The promise of hematopoietic stem cell transplantation for autoimmune diseases. *Bone Marrow Transplant. 31:521.*
3. Teshima, T., and J. L. Ferrara. 2002. Understanding the alloresponse: new approaches to graft-versus-host disease prevention. *Semin. Hematol. 39:15.*
4. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol. 136:2348.*
5. Blazar, B. R., P. A. Taylor, S. Smith, and D. A. Vallera. 1995. Interleukin-10 administration decreases survival in murine recipients of major histocompatibility complex disparate donor bone marrow grafts. *Blood 85:842.*
6. Atkinson, K., C. Matias, A. Guiffre, R. Seymour, M. Cooley, J. Biggs, V. Munro, and S. Gillis. 1991. In vivo administration of granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF, interleukin-1 (IL-1), and IL-4, alone and in combination, after allogeneic murine hematopoietic stem cell transplantation. *Blood 77:1376.*
7. Krenger, W., K. Snyder, S. Smith, and J. L. Ferrara. 1994. Effects of exogenous interleukin-10 in a murine model of graft-versus-host disease to minor histocompatibility antigens. *Transplantation 58:1251.*
8. Nikolic, B., S. Lee, R. T. Bronson, M. J. Grusby, and M. Sykes. 2000. Th1 and Th2 mediate acute graft-versus-host disease, each with distinct end-organ targets. *J. Clin. Invest. 105:1289.*
9. Hill, G. R., and J. L. Ferrara. 2000. The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation. *Blood 95:2754.*
10. Krenger, W., K. M. Snyder, J. C. Byon, G. Falzarano, and J. L. Ferrara. 1995. Polarized type 2 alloreactive $CD4^+$ and $CD8^+$ donor T cells fail to induce experimental acute graft-versus-host disease. *J. Immunol. 155:585.*

11. Krenger, W., K. R. Cooke, J. M. Crawford, S. T. Sonis, R. Simmons, L. Pan, J. Delmonte, Jr., M. Karandikar, and J. L. Ferrara. 1996. Transplantation of polarized type 2 donor T cells reduces mortality caused by experimental graft-versus-host disease. *Transplantation 62:1278.*
12. Fowler, D. H., K. Kurasawa, R. Smith, M. A. Eckhaus, and R. E. Gress. 1994. Donor CD4-enriched cells of Th2 cytokine phenotype regulate graft-versus-host disease without impairing allogeneic engraftment in sublethally irradiated mice. *Blood 84:3540.*
13. Reid, S. D., G. Penna, and L. Adorini. 2000. The control of T cell responses by dendritic cell subsets. *Curr. Opin. Immunol. 12:114.*
14. O'Garra, A. 1998. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity 8:275.*
15. Kronenberg, M., and L. Gapin. 2002. The unconventional lifestyle of NKT cells. *Nat. Rev. Immunol. 2:557.*
16. Godfrey, D. I., H. R. MacDonald, M. Kronenberg, M. J. Smyth, and L. Van Kaer. 2004. NKT cells: what's in a name? *Nat. Rev. Immunol. 4:231.*
17. Benlagha, K., A. Weiss, A. Beavis, L. Teyton, and A. Bendelac. 2000. In vivo identification of glycolipid antigen-specific T cells using fluorescent CD1d tetramers. *J. Exp. Med. 191:1895.*
18. Taniguchi, M., M. Harada, S. Kojo, T. Nakayama, and H. Wakao. 2003. The regulatory role of Vα14 NKT cells in innate and acquired immune response. *Annu. Rev. Immunol. 21:483.*
19. Yoshimoto, T., A. Bendelac, C. Watson, J. Hu-Li, and W. E. Paul. 1995. Role of NK1.1$^+$ T cells in a TH2 response and in immunoglobulin E production. *Science 270:1845.*
20. Bendelac, A., R. D. Hunziker, and O. Lantz. 1996. Increased interleukin 4 and immunoglobulin E production in transgenic mice overexpressing NK1 T cells. *J. Exp. Med. 184:1285.*
21. Smiley, S. T., M. H. Kaplan, and M. J. Grusby. 1997. Immunoglobulin E production in the absence of interleukin-4-secreting CD1-dependent cells. *Science 275:977.*
22. Mendiratta, S. K., W. D. Martin, S. Hong, A. Boesteanu, S. Joyce, and L. Van Kaer. 1997. CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. *Immunity 6:469.*
23. Chen, Y. H., N. M. Chiu, M. Mandal, N. Wang, and C. R. Wang. 1997. Impaired NK1$^+$ T cell development and early IL-4 production in CD1-deficient mice. *Immunity 6:459.*
24. Bendelac, A. 1995. Positive selection of mouse NK1$^+$ T cells by CD1-expressing cortical thymocytes. *J. Exp. Med. 182:2091.*
25. Coles, M. C., and D. H. Raulet. 2000. NK1.1$^+$ T cells in the liver arise in the thymus and are selected by interactions with class I molecules on CD4$^+$CD8$^+$ cells. *J. Immunol. 164:2412.*
26. Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, H. Koseki, and M. Taniguchi. 1997. CD1d-restricted and TCR-mediated activation of vα14 NKT cells by glycosylceramides. *Science 278:1626.*
27. Miyamoto, K., S. Miyake, and T. Yamamura. 2001. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. *Nature 413:531.*
28. Wall, D. A., S. D. Hamberg, D. S. Reynolds, S. J. Burakoff, A. K. Abbas, and J. L. Ferrara. 1988. Immunodeficiency in graft-versus-host disease. I. Mechanism of immune suppression. *J. Immunol. 140:2970.*
29. Cooke, K. R., L. Kobzik, T. R. Martin, J. Brewer, J. Delmonte, Jr., J. M. Crawford, and J. L. Ferrara. 1996. An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation. I. The roles of minor H antigens and endotoxin. *Blood 88:3230.*
30. Kobayashi, E., K. Motoki, T. Uchida, H. Fukushima, and Y. Koezuka. 1995. KRN7000, a novel immunomodulator, and its antitumor activities. *Oncol. Res. 7:529.*
31. Oki, S., A. Chiba, T. Yamamura, and S. Miyake. 2004. The clinical implication and molecular mechanism of preferential IL-4 production by modified glycolipid-stimulated NKT cells. *J. Clin. Invest. 113:1631.*
32. Teshima, T., N. Mach, G. R. Hill, L. Pan, S. Gillessen, G. Dranoff, and J. L. Ferrara. 2001. Tumor cell vaccine elicits potent antitumor immunity after allogeneic T-cell-depleted bone marrow transplantation. *Cancer Res. 61:162.*
33. Hill, G. R., K. R. Cooke, T. Teshima, J. M. Crawford, J. C. Keith, Jr., Y. S. Brinson, D. Bungard, and J. L. Ferrara. 1998. Interleukin-11 promotes T cell polarization and prevents acute graft-versus-host disease after allogeneic bone marrow transplantation. *J. Clin. Invest. 102:115.*
34. Takeda, K., T. Tanaka, W. Shi, M. Matsumoto, M. Minami, S. Kashiwamura, K. Nakanishi, N. Yoshida, T. Kishimoto, and S. Akira. 1996. Essential role of Stat6 in IL-4 signalling. *Nature 380:627.*
35. Shimoda, K., J. van Deursen, M. Y. Sangster, S. R. Sarawar, R. T. Carson, R. A. Tripp, C. Chu, F. W. Quelle, T. Nosaka, D. A. Vignali, et al. 1996. Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. *Nature 380:630.*
36. Zeng, D., D. Lewis, S. Dejbakhsh-Jones, F. Lan, M. Garcia-Ojeda, R. Sibley, and S. Strober. 1999. Bone marrow NK1.1$^-$ and NK1.1$^-$ T cells reciprocally regulate acute graft versus host disease. *J. Exp. Med. 189:1073.*
37. Teshima, T., R. Ordemann, P. Reddy, S. Gagin, C. Liu, K. R. Cooke, and J. L. Ferrara. 2002. Acute graft-versus-host disease does not require alloantigen expression on host epithelium. *Nat. Med. 8:575.*
38. Reiner, S. L. 2001. Helper T cell differentiation, inside and out. *Curr. Opin. Immunol. 13:351.*
39. Farrar, J. D., H. Asnagli, and K. M. Murphy. 2002. T helper subset development: roles of instruction, selection, and transcription. *J. Clin. Invest. 109:431.*
40. Lan, F., D. Zeng, M. Higuchi, P. Huie, J. P. Higgins, and S. Strober. 2001. Predominance of NK1.1$^+$TCRαβ$^+$ or DX5$^+$TCRαβ$^+$ T cells in mice conditioned with fractionated lymphoid irradiation protects against graft-versus-host disease: "natural suppressor" cells. *J. Immunol. 167:2087.*
41. Rigby, S. M., T. Rouse, and E. H. Field. 2003. Total lymphoid irradiation non-myeloablative preconditioning enriches for IL-4-producing CD4$^+$-TNK cells and skews differentiation of immunocompetent donor CD4$^+$ cells. *Blood 101:2024.*
42. Lan, F., D. Zeng, M. Higuchi, J. P. Higgins, and S. Strober. 2003. Host conditioning with total lymphoid irradiation and antithymocyte globulin prevents graft-versus-host disease: the role of CD1-reactive natural killer T cells. *Biol. Blood Marrow Transplant. 9:355.*
43. Morita, Y., J. Yang, R. Gupta, K. Shimizu, E. A. Shelden, J. Endres, J. J. Mule, K. T. McDonagh, and D. A. Fox. 2001. Dendritic cells genetically engineered to express IL-4 inhibit murine collagen-induced arthritis. *J. Clin. Invest. 107:1275.*
44. Reddy, P., T. Teshima, G. Hildebrandt, D. L. Williams, C. Liu, K. R. Cooke, and J. L. Ferrara. 2003. Pretreatment of donors with interleukin-18 attenuates acute graft-versus-host disease via STAT6 and preserves graft-versus-leukemia effects. *Blood 101:2877.*
45. Fowler, D. H., and R. E. Gress. 2000. Th2 and Tc2 cells in the regulation of GVHD, GVL, and graft rejection: considerations for the allogeneic transplantation therapy of leukemia and lymphoma. *Leuk. Lymphoma 38:221.*
46. Teshima, T., G. R. Hill, L. Pan, Y. S. Brinson, M. R. van den Brink, K. R. Cooke, and J. L. Ferrara. 1999. IL-11 separates graft-versus-leukemia effects from graft-versus-host disease after bone marrow transplantation. *J. Clin. Invest. 104:317.*
47. Fernando, G. J., T. J. Stewart, R. W. Tindle, and I. H. Frazer. 1998. Th2-type CD4$^+$ cells neither enhance nor suppress antitumor CTL activity in a mouse tumor model. *J. Immunol. 161:2421.*
48. Pan, L., J. Delmonte, Jr., C. K. Jalonen, and J. L. Ferrara. 1995. Pretreatment of donor mice with granulocyte colony-stimulating factor polarizes donor T lymphocytes toward type-2 cytokine production and reduces severity of experimental graft-versus-host disease. *Blood 86:4422.*
49. Morecki, S., S. Panigrahi, G. Pizov, E. Yacovlev, Y. Gelfand, O. Eizik, and S. Slavin. 2004. Effect of KRN7000 on induced graft-versus-host disease *Exp. Hematol. 32:630.*
50. Rogers, P. R., A. Matsumoto, O. Naidenko, M. Kronenberg, T. Mikayama, and S. Kato. 2004. Expansion of human Vα24$^+$ NKT cells by repeated stimulation with KRN7000. *J. Immunol. Methods 285:197.*
51. Giaccone, G., C. J. Punt, Y. Ando, R. Ruijter, N. Nishi, M. Peters, B. M. von Blomberg, R. J. Scheper, H. J. van der Vliet, A. J. van den Eertwegh, et al. 2002. A phase I study of the natural killer T-cell ligand α-galactosylceramide (KRN7000) in patients with solid tumors. *Clin. Cancer Res. 8:3702.*

Modulation of CD1d-restricted NKT cell responses by using *N*-acyl variants of α-galactosylceramides

Karl O. A. Yu*†, Jin S. Im*†, Alberto Molano*†, Yves Dutronc*‡, Petr A. Illarionov§, Claire Forestier*, Nagatoshi Fujiwara*¶, Isa Arias*, Sachiko Miyake‖, Takashi Yamamura‖, Young-Tae Chang**, Gurdyal S. Besra§, and Steven A. Porcelli*††

*Department of Microbiology and Immunology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461; §School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom; ‖Department of Immunology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan; and **Department of Chemistry, New York University, 29 Washington Place, New York, NY 10003

Edited by Douglas T. Fearon, University of Cambridge, Cambridge, United Kingdom, and approved January 18, 2005 (received for review October 8, 2004)

A form of α-galactosylceramide, KRN7000, activates CD1d-restricted Vα14-invariant (Vα14i) natural killer (NK) T cells and initiates multiple downstream immune reactions. We report that substituting the C26:0 *N*-acyl chain of KRN7000 with shorter, unsaturated fatty acids modifies the outcome of Vα14i NKT cell activation. One analogue containing a diunsaturated C20 fatty acid (C20:2) potently induced a T helper type 2-biased cytokine response, with diminished IFN-γ production and reduced Vα14i NKT cell expansion. C20:2 also exhibited less stringent requirements for loading onto CD1d than KRN7000, suggesting a mechanism for the immunomodulatory properties of this lipid. The differential cellular response elicited by this class of Vα14i NKT cell agonists may prove to be useful in immunotherapeutic applications.

cytokines | inflammation | autoimmunity | immunoregulation

Natural killer (NK) T cells were defined originally as lymphocytes coexpressing T cell receptors (TCRs) and C-type lectin receptors characteristic of NK cells. A major subset of NKT cells recognizes the MHC class I-like molecule CD1d by using TCRs composed of an invariant TCR-α chain (mouse Vα14-Jα18, human Vα24-Jα18) paired with TCR-β chains with markedly skewed Vβ usage (1). These CD1d-restricted Vα14-invariant (Vα14i) NKT cells are highly conserved in phenotype and function between mice and humans (2). Vα14i NKT cells influence various immune responses and play an important role in regulating autoimmunity (3, 4). One example is the nonobese diabetic mouse. When compared with normal mice, nonobese diabetic mice have fewer Vα14i NKT cells, which are defective in their capacity to produce antiinflammatory cytokines like IL-4 (5, 6). Deficiencies in NKT cells have also been observed in humans with various autoimmune diseases (7, 8).

Vα14i NKT cells have been manipulated to prevent or treat autoimmune disease, mostly through the use of KRN7000, a synthetic α-galactosylceramide (α-GalCer, Fig. 1*A*) that binds to the hydrophobic groove of CD1d and then activates Vα14i NKT cells by means of TCR recognition (9). KRN7000 treatment of nonobese diabetic mice blocks development of T helper (T_H) type 1-mediated autoimmune destruction of pancreatic islet β-cells, thus delaying or preventing disease (10–12). There has been considerable interest in methods that would allow a more selective activation of these cells. In particular, the ability to trigger IL-4 production without eliciting strong IFN-γ or other proinflammatory cytokines may reinforce the immunoregulatory functions of Vα14i NKT cells. This effect is detected after Vα14i NKT cell activation with a glycolipid designated OCH, which is an α-GalCer analogue that is structurally distinct from KRN7000 in having a substantially shorter sphingosine chain and functionally by its preferential induction of IL-4 secretion (13, 14).

In this study, we investigated responses to α-GalCer analogues produced by alteration of the length and extent of unsaturation of their *N*-acyl substituents. Such modifications altered the outcome of Vα14i NKT cell activation and, in some cases, led to a T_H2-biased and potentially antiinflammatory cytokine response. This change in the NKT cell response was likely the result of an alteration of downstream steps in the cascade of events triggered by Vα14i NKT cell activation, including the reduction of secondary activation of IFN-γ-producing NK cells. These findings point to a class of Vα14i NKT cell agonists that may have superior properties for the treatment of autoimmune and inflammatory diseases.

Materials and Methods

Mice and Cell Lines. C57BL/6 mice (8- to 15-wk-old females) were obtained either from The Jackson Laboratory or Taconic Farms. $CD1d^{-/-}$ mice were provided by M. Exley and S. Balk (Beth Israel–Deaconess Medical Center, Harvard Medical School, Boston) (15). Vα14i NKT cell-deficient $Jα18^{-/-}$ mice were a gift from M. Taniguchi and T. Nakayama (Chiba University, Chiba, Japan) (16). Both knockout mice were in the C57BL/6 background. Animals were kept in specific pathogen-free housing. The protocols that we used were in accordance with approved institutional guidelines.

Mouse CD1d-transfected RMA-S cells (RMA-S.mCD1d) were provided by S. Behar (Brigham and Women's Hospital, Harvard Medical School) (17). WT or cytoplasmic tail-deleted CD1d-transfected A20 cells and the Vα14i NKT hybridoma DN3A4-1.2 were provided by M. Kronenberg (La Jolla Institute for Allergy and Immunology, La Jolla, CA) (18, 19). Hybridoma DN32D3 was a gift from A. Bendelac (University of Chicago, Chicago) (1). Cells were cultured in RPMI medium 1640 (GIBCO) supplemented with 10% heat-inactivated FCS (Gemini Biological Products, Calabasas, CA)/10 mM Hepes/2 mM L-glutamine/0.1 mM nonessential amino acids/55 μM 2-mercaptoethanol/100 units/ml penicillin/100 μg/ml streptomycin (GIBCO) in a 37°C humidified incubator with 5% CO_2.

Glycolipids. BF1508-84 was synthesized by Biomira (Edmonton, Canada). OCH [(2*S*, 3*S*, 4*R*)-1-*O*-(α-D-galactopyranosyl)-*N*-tetracosanoyl-2 amino-1,3,4-nonanetriol] was synthesized as described (13). An overview of the methods for synthesis of KRN7000 [(2*S*, 3*S*, 4*R*)-1-*O*-(α-D-galactopyranosyl)-*N*-hexaco-

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: Vα14i, Vα14 invariant; NK, natural killer; α-GalCer, α-galactosylceramide; T_H, T helper; TCR, T cell receptor; RMA-S.mCD1d, mouse CD1d-transfected RMA-S cells.

†K.O.A.Y., J.S.I., and A.M. contributed equally to this work.

‡Present address: Department of Dermatology, Bocage Hospital, BP 77908, 21079 Dijon Cedex, France.

¶Present address: Department of Host Defense, Graduate School of Medicine, Osaka City University, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan.

††To whom correspondence should be addressed. E-mail: porcelli@aecom.yu.edu.

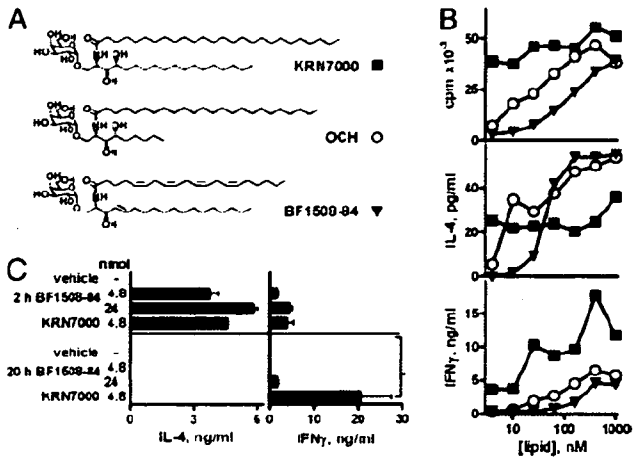

Fig. 1. Induction of a T_H2-polarized cytokine response by an unsaturated analogue of α-GalCer. (*A*) Glycolipid structures. (*B*) [^{3}H]thymidine incorporation and supernatant IL-4 and IFN-γ levels in 72-h splenocyte cultures with graded amounts of glycolipid. Means from triplicate cultures are shown; SEMs were typically < 10% of the mean. (*C*) Serum IL-4 and IFN-γ levels (at 2 and 20 h) of C57BL/6 mice injected i.p. with 4.8 or 24 nmol of glycolipid. KRN7000 was the only glycolipid that induced significant IFN-γ levels at 20 h (*, $P < 0.05$, Kruskall–Wallis test, Dunn's posttest). Means ± SD of two or three mice per group are shown.

sanoyl-2-amino-1,3,4-octadecanetriol] and other *N*-acyl analogues used in this study is shown in Fig. 7, which is published as supporting information on the PNAS web site. Lipids were dissolved in chloroform/methanol (2:1 ratio) and stored at −20°C. Aliquots from this stock were dried and reconstituted to either 100 μM in DMSO for *in vitro* work or to 500 μM in 0.5% Tween-20 in PBS for *in vivo* studies.

***In Vitro* Stimulations.** Bulk splenocytes were plated at 300,000 cells per well in 96-well flat-bottom tissue culture plates with glycolipid diluted in 200 μl of medium. After 48 or 72 h at 37°C, 150 μl of supernatant was removed for cytokine measurements, and 0.5 μCi (1 Ci = 37 GBq) [^{3}H]thymidine per well (specific activity 2 Ci/mmol; PerkinElmer) was added for an 18-h pulse. Proliferation was estimated by harvesting cells onto 96-well filter mats and counting β-scintillations with a 1450 Microbeta Trilux (Wallac, Gaithersburg, MD; PerkinElmer).

Supernatant levels of IL-2, IL-4, IL-12p70, and IFN-γ were measured by ELISA using capture and biotinylated detection antibody pairs (BD PharMingen) and streptavidin–horseradish peroxidase (Zymed) with TMB-Turbo substrate (Pierce) or streptavidin–alkaline phosphatase (Zymed) with 4-nitrophenyl phosphate substrate (Sigma). IL-2 standard was obtained from R & D Systems; IL-4, IL-12p70 and IFN-γ were obtained from PeproTech (Rocky Hill, NJ).

Hybridoma Stimulations. CD1d$^+$ RMA-S or A20 cells (50,000 cells in 100 μl per well) were pulsed with graded doses of glycolipid for 6 h at 37°C. After three washes in PBS, Vα14i NKT hybridoma cells (50,000 cells in 100 μl) were added for 12 h. Supernatant IL-2 was assayed by ELISA. Alternatively, CD1d-transfected cells (RMA-S.mCD1d) were lightly fixed either before or after exposure to antigen (20). Cells were washed twice in PBS and then fixed in 0.05% glutaraldehyde (grade I, Sigma) in PBS for 30 s at room temperature. Fixative was quenched by addition of 0.2 M L-lysine (pH 7.4) for 2 min, followed by two washes with medium before addition of responders.

For cell-free presentation, recombinant mouse CD1d (1 μg/ml in PBS) purified from a baculovirus expression system (21) was adhered to tissue culture plates for 1 h at 37°C. After the washing off of unbound protein, glycolipids were then added at varying concentrations for 1 h at 37°C. Lipids were added in a 150 mM NaCl/10 mM sodium phosphate buffer (pH 7) with or without 0.025% Triton X-100. Wells were washed before addition of hybridoma cells.

***In Vivo* Studies.** Mice were given i.p. injections of 4.8 nmol of glycolipid in 0.2 ml of PBS plus 0.025% Tween-20 or vehicle alone. Sera were collected and tested for IL-4, IL-12p70, and IFN-γ, as described above. Alternatively, mice were killed at various times for FACS analysis.

Flow Cytometry. Splenocytes or thymocytes were isolated and used without further purification. Nonspecific staining was blocked by using FACS buffer (0.1% BSA/0.05% NaN_3 in PBS) with 10 μg/ml rat anti-mouse CD16/32 (2.4G2; The American Type Culture Collection). Cells (≤10^6) were stained with phycoerythrin or allophycocyanin-conjugated glycolipid/mouse CD1d tetramers (21) for 30–90 min at room temperature and then with fluorescently labeled antibodies (from Caltag, South San Francisco, CA, or PharMingen) for 30 min at 4°C. Data were acquired on either a FACSCalibur or LSR-II flow cytometer (Becton Dickenson) and analyzed by using WINMDI 2.8 (Scripps Research Institute, La Jolla, CA). For some experiments, dead cells were excluded by using propidium iodide (Sigma) or 4′,6-diamidino-2-phenylindole (Roche).

FACS-based cytokine secretion assays (Miltenyi Biotec, Auburn, CA) were used to quantitatively detect single-cell production of IL-4 or IFN-γ. Splenocytes were aseptically collected from mice that were previously injected i.p. with glycolipid analogues and not subjected to further stimulation. When applicable, 10^6 cells were prestained with labeled tetramer for 30 min at room temperature and then washed in PBS plus 0.1% BSA. Cells were then stained with the cytokine catch reagent according to the manufacturer's instructions, followed by incubation with rotation in 2 ml of medium at 37°C for 45 min. Cells were then washed, stained with fluorescently labeled antibodies to cell-surface antigens, phycoerythrin-conjugated anti-IFN-γ or IL-4, and propidium iodide, as described above.

Results

T_H2-Skewing Properties of an α-GalCer Analogue. During screening of a panel of synthetic glycosyl ceramides, we identified a compound that showed T_H2-skewing of the cytokine profile generated by Vα14i NKT cell activation. Glycolipid BF1508-84 differed structurally from both OCH and KRN7000 by having a shortened, unsaturated fatty-acid chain (C20:4 arachidonate) and a double bond in place of the 4-hydroxy in the sphingosine base (Fig. 1*A*). Despite these modifications, BF1508-84 activated proliferation and cytokine secretion by mouse splenocytes (Fig. 1*B*). These responses were Vα14i NKT cell-dependent, as demonstrated by their absence in both CD1d$^{-/-}$ and Jα18$^{-/-}$ mice (data not shown). Maximal proliferation and IL-4 levels were comparable with those obtained with KRN7000 and OCH, although a higher concentration of BF1508-84 was required to reach similar responses. Interestingly, IFN-γ secretion stimulated by BF1508-84, even at higher tested concentrations, did not reach the levels seen with KRN7000. This profile of cytokine responses suggested that BF1508-84 can elicit a T_H2-biased Vα14i NKT cell-dependent cytokine production, similar to OCH (13).

We measured serum cytokine levels at various times after a single injection of either KRN7000 or BF1508-84 into C57BL/6 mice. Our studies confirm published reports that a single i.p. injection of KRN7000 leads to a rapid 2-h peak of serum IL-4 (Fig. 1*C* and data not shown). However, IFN-γ levels were relatively low at 2 h but rose to a plateau at 12–24 h (13, 22). With

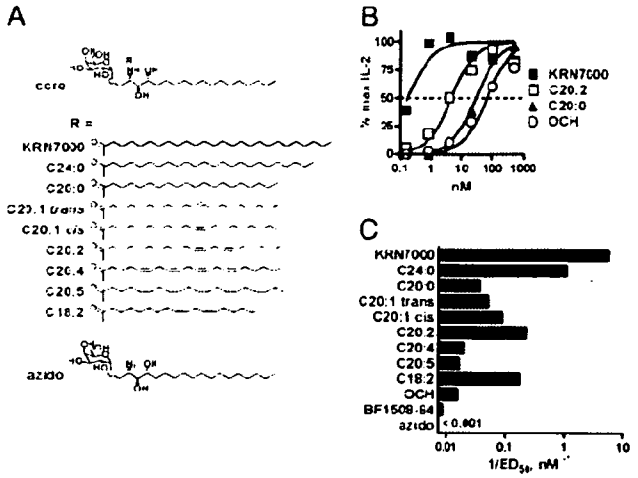

Fig. 2. Recognition of a panel of unsaturated analogues of KRN7000 by a canonical Vα14i NKT hybridoma. (*A*) Analogue structures. (*B*) Dose–response curves showing IL-2 production by hybridoma DN3A4–1.2 after stimulation with RMA-S.mCD1d cells pulsed with various doses of glycolipid. Maximal IL-2 concentrations in each assay were designated as 100%. Four-parameter logistic equation dose–response curves are shown; the dotted line denotes the half-maximal dose. (*C*) Relative potencies of the analogue panel in Vα14i NKT cell recognition, plotted as the reciprocal of the effective dose required to elicit a half-maximal response ($1/ED_{50}$). Similar results were obtained by using another Vα14i NKT hybridoma, DN32D3.

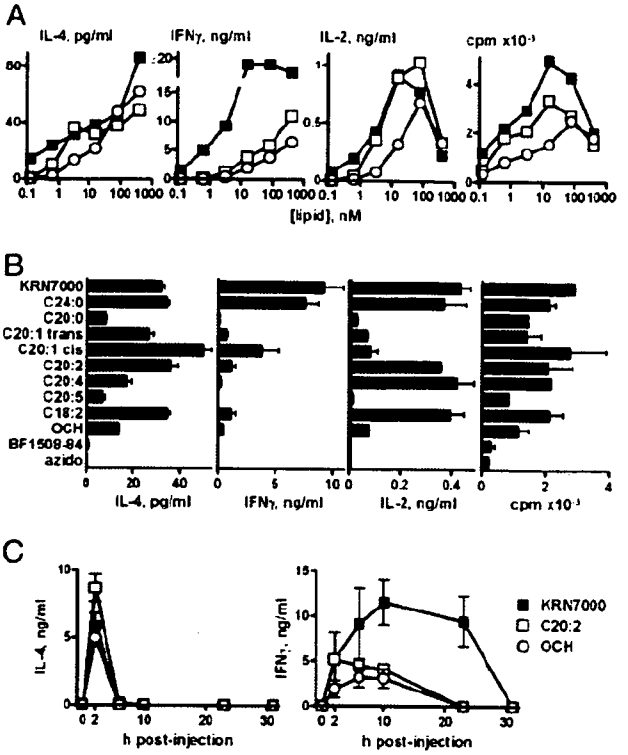

Fig. 3. T_H2-skewing of *in vitro* and *in vivo* cytokine responses to C20:2. (*A*) Dose–response curves reporting 48 h IL-4, IFN-γ, or IL-2 production, and cell proliferation of splenocytes in response to KRN7000, C20:2, and OCH. Means of duplicate cultures are shown; SEM were <10% of the means. (*B*) Cytokine and proliferation measurements on splenocytes exposed to a submaximal dose (3.2 nM) of the panel of α-GalCer analogues shown in Fig. 2. Mean ± SEM from duplicate cultures shown. (*C*) Serum IL-4 and IFN-γ levels in mice given 4.8 nmol of KRN7000, C20:2, or OCH. Mean ± SD of two or three mice are shown. Vehicle-treated mice had cytokine levels below limits of detection. The results shown are representative of two or more experiments.

BF1508-84, production of IL-4 at 2 h was preserved, whereas IFN-γ was barely detectable at 20 h (Fig. 1*C*). This pattern was identical to that reported for OCH (13, 22) and was not due to the lower potency of BF1508-84 because a 5-fold greater dose did not change the T_H2-biased cytokine profile (Fig. 1*C*).

Systematic Variation of Fatty-Acyl Unsaturation in α-GalCer. The cytokine response to BF1508-84 suggested that altering the fatty-acid length and unsaturation of α-GalCer could provide an effective strategy for creating Vα14i NKT cell activators with modified functional properties. We used a synthetic approach (Fig. 7, and G.S.B. and P.A.I., unpublished data) to generate lipids in which 20-carbon acyl chains with varying degrees of unsaturation were coupled onto the α-galactosylated sphingosine core structure (Fig. 2*A*). These compounds were first screened for the ability to activate a canonical Vα14-Jα18/Vβ8.2$^+$, CD1d-restricted NKT cell hybridoma cocultured with CD1d$^+$ antigen-presenting cells. Hybridoma DN3A4–1.2 recognized all C20 analogues of α-GalCer with various potencies when presented by CD1d-transfected RMA-S cells, and it failed to recognize an azido-substituted analogue lacking a fatty-acid chain (Fig. 2 *B* and *C*). As reported (9), mere shortening of the fatty-acid chain affected Vα14i NKT cell recognition, and reduction of saturated fatty-acid length from C26 to C20 was associated with a ≈2 log decrease in potency. However, insertion of double bonds into the C20 acyl chain augmented stimulatory activity. One lipid in particular, with unsaturations at carbons 11 and 14 (C20:2), was more potent than other analogues in the panel. This increase in potency seemed to be a direct result of the two double bonds, because an independently synthesized analogue with a slightly shorter diunsaturated acyl chain (C18:2) showed a potency similar to that of C20:2 (Fig. 2*C*).

We also studied *in vitro* splenocyte cytokine polarization resulting from Vα14i NKT cell stimulation by each lipid in the panel. Supernatant IL-4, IFN-γ, and IL-2 levels were measured over a wide range of glycolipid concentrations. All C20 variants induced IL-4 production comparable with that of KRN7000 (Fig. 3 *A* and *B*, and data not shown). However, IFN-γ levels for all but one C20 analogue (C20:1 *cis*) were markedly reduced to one-fourth of the maximal levels observed with KRN7000 and the closely related C24:0 analogue, or less. In addition, C20:1-*cis*, C20:2, and C18:2 were unique in this class of compounds in inducing strong IL-2 production and cellular proliferation similar to that seen with KRN7000 and C24:0 yet with much lower IFN-γ induction. This *in vitro* T_H2-bias was also evident *in vivo*. Mice given C20:2 and C20:4 showed systemic cytokine production that resembled stimulation by OCH or BF1508-84. Thus, a rapid burst of serum IL-4 was observed without the delayed and sustained production of IFN-γ typical of KRN7000 (Fig. 3*C* and data not shown). No significant difference between the glycolipids was seen in serum IL-12p70 levels at 6 h after treatment (data not shown).

Identification of Cytokine-Producing Cells *in Vivo*. Previous reports (23–25) established that Vα14i NKT cells are a predominant source of IL-4 and IFN-γ in the early (2 h) response to KRN7000 and that by 6 h after injection these cells become progressively undetectable because of receptor down-modulation, whereas secondarily activated NK cells begin to actively produce IFN-γ. Gating on either α-GalCer-loaded CD1d tetramer$^+$ or NK1.1$^+$ T cells, we observed similar strong cytokine secretion for both IL-4 (data not shown) and IFN-γ in Vα14i NKT cells at 2 h after injection of KRN7000 or C20:2 (Fig. 4 *A* and *B*). We concluded

IMMUNOLOGY

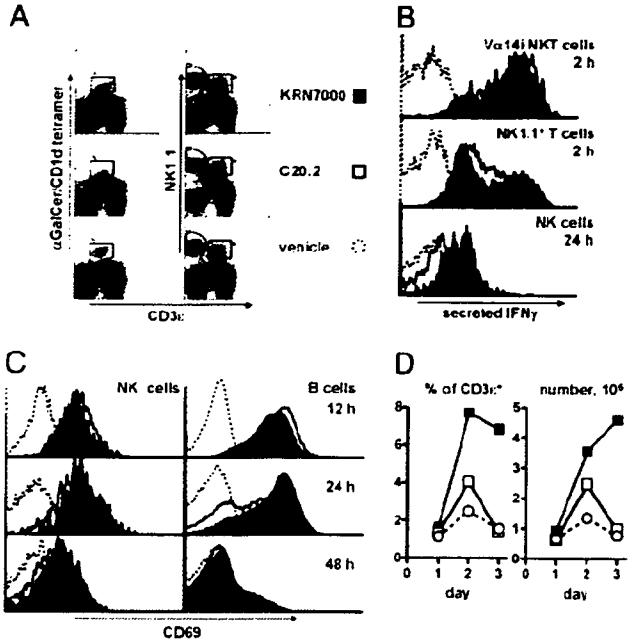

Fig. 4. Sequelae of KRN7000 and C20:2-induced Vα14i NKT cell activation. (*A*) Vα14i NKT cell (tetramer$^+$ CD3ε^{int}), NK cell (NK1.1$^+$ CD3ε$^-$), and NK1.1$^+$ T cell (NK1.1^{int} CD3ε^{int}) identification by FACS in splenocytes from mice given KRN7000, C20:2, or vehicle i.p. 2 h earlier. Lymphocytes gated as negative for B220 and propidium iodide are shown. (*B*) Histogram profiles for IFN-γ secretion of splenic Vα14i NKT, NK1.1$^+$ T, or NK cells from mice 2 or 24 h after treatment with glycolipid. IFN-γ-staining in C24:0-stimulated samples was identical to that of KRN7000-stimulated samples. (*C*) CD69 levels of splenic NK cells (gated as CD3ε$^-$ NK1.1$^+$) or B cells (CD3ε$^-$ NK1.1$^-$ B220$^+$) at 12, 24, or 48 h after injection of glycolipid. (*D*) Splenic Vα14i NKT cell (B220$^-$ CD3ε^{int} tetramer$^+$) frequency, measured as either percentages of T cells or as total NKT cell number, in mice 1, 2, or 3 days after glycolipid administration. The results shown are representative of three independent experiments.

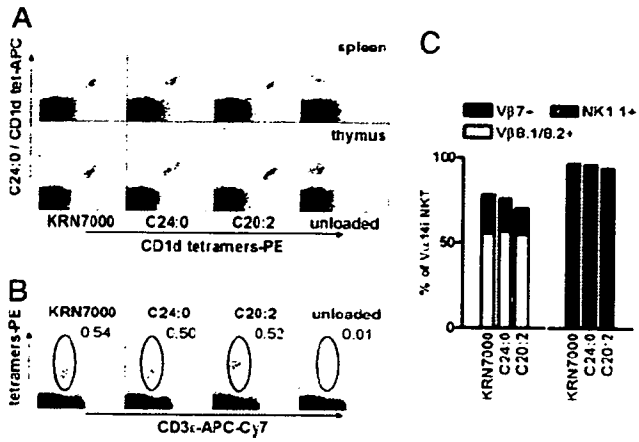

Fig. 5. Recognition of KRN7000, C24:0, and C20:2 by the same population of Vα14i NKT cells. (*A*) Costaining of C57BL/6 splenocytes or thymocytes with allophycocyanin-conjugated CD1d tetramers assembled with C24:0, and phycoerythrin-labeled CD1d tetramers assembled with various analogues. (*B*) Thymocytes were stained with C24:0, C20:2, KRN7000, or vehicle-loaded CD1d tetramers–phycoerythrin, and with antibodies to B220, CD3ε, Vβ7, Vβ8.1/8.2, or NK1.1. Dot plots show gating for tetramer$^+$ T cells, after exclusion of B lymphocytes, and dead cells. (*C*) TCR Vβ and NK1.1 phenotype of tetramer$^+$ CD3ε^{int} thymocytes. Analogous results were obtained with splenocytes. The results shown are representative of three or more experiments.

that cytokine polarization observed after C20:2 administration was not due to differences in the initial Vα14i NKT cell response but, rather, reflected altered downstream events such as the relatively late IFN-γ production by activated NK cells.

Secreted cytokine staining confirmed that in both KRN7000- and C20:2-treated mice, NK cells were IFN-γ$^+$ at 6–12 h after treatment (26, 27). However, whereas splenic NK cells from mice that received either KRN7000 or the closely related C24:0 analogue strongly produced IFN-γ as late as 24 h after initial activation, NK cells from C20:2-treated mice showed substantially reduced staining (Fig. 4*B*). Together, these results pointed to a less sustained secondary IFN-γ production by NK cells (rather than a change in the initial cytokine response of Vα14i NKT cells) as the major factor responsible for the T_H2 bias of the systemic cytokine response to C20:2.

Sequelae of Vα14i NKT Cell Activation by C20:2. Secondary activation of bystander B and NK cells after KRN7000 administration has been studied by using expression of the activation marker CD69 (26, 28–30). We followed CD69 expression of splenic NK and B cell populations for several hours after KRN7000 or C20:2 administration. Both populations began to up-regulate CD69 at 4–6 h after injection (data not shown). Paradoxically, C20:2 induced slightly higher CD69 levels on both cell populations up until 12 h, although this trend was reversed from 24 h onwards, suggesting an earlier up-regulation yet faster subsequent down-regulation of the marker (Fig. 4*C*). NK cell forward scatter likewise remained higher in KRN7000-treated mice at days 1–3 compared with C20:2-treated mice (data not shown).

It is established that Vα14i NKT cells expand beyond homeostatic levels 2 or 3 days after KRN7000 stimulation (24, 25). In our study, a 3- to 5-fold expansion in splenic Vα14i NKT cell number occurred in KRN7000-treated mice at day 3 after injection. Interestingly, after *in vivo* administration of C20:2, only a minimal transient expansion was observed on day 2, with no expansion of the Vα14i NKT cell population thereafter, even as late as day 5 (Fig. 4*D* and data not shown). Together, our findings indicated pronounced alterations in the late sequelae of Vα14i NKT cell activation with the C20:2 analogue compared with KRN7000.

Recognition of KRN7000 and C20:2 by Identical Cell Populations. CD1d complexes containing the α-GalCer analogue OCH have been shown to have significantly reduced avidity for TCRs of Vα14i NKT cells compared with binding of KRN7000-loaded complexes (31). This finding suggests the possibility that the T_H2-biased response of C20:2 could be a result of preferential stimulation of Vα14i NKT cell subsets with TCRs of higher affinity for lipid-loaded CD1d. In fact, phenotypically defined subsets of murine and human NKT cells have been described that show a bias toward increased production of IL-4 relative to IFN-γ upon stimulation (32–36). However, by costaining of splenic and thymic Vα14i NKT cells by using CD1d tetramers loaded with different lipids, we demonstrated that identical populations recognized C24:0, C20:2, and KRN7000 (Fig. 5*A*). Single staining with these reagents revealed no difference in Vβ usage or NK1.1 status of cells reactive with the different analogue tetramers (Fig. 5 *B* and *C*). Interestingly, C20:2-loaded tetramers stained NKT cells more strongly than tetramers loaded with KRN7000, reflecting a slightly higher affinity of the C20:2–CD1d complex to the Vα14i TCR (J.S.I. and S.A.P., unpublished results). Together, these findings demonstrated that the altered cytokine response to C20:2 cannot be the result of preferential activation of a subset of Vα14i NKT cells.

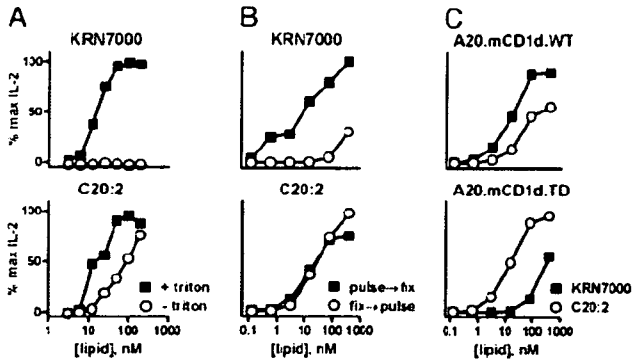

Fig. 6. Differential requirements for CD1d loading with KRN7000 and C20:2. IL-2 response of hybridoma DN3A4–1.2 to glycolipid presentation in three *in vitro* CD1d presentation systems: platebound CD1d loaded with varying amounts of KRN7000 or C20:2 in the presence or absence of the detergent Triton X-100 (*A*), RMA-S.CD1d cells pulsed with glycolipid before or after glutaraldehyde fixation (*B*), or WT or cytoplasmic tail-deleted (TD) CD1d-transfected A20 cells, loaded with either KRN7000 or C20:2 (*C*).

Loading Requirements of α-GalCer Analogues onto CD1d. To find an alternative explanation for the T_H2-biased response to C20:2, we studied requirements for handling of different forms of α-GalCer by antigen-presenting cells. We employed a cell-free system in which platebound mouse CD1d was loaded with doses of KRN7000 or C20:2 in the presence or absence of the detergent Triton X-100 (37). By using IL-2 production by DN3A4–1.2 as a readout for glycolipid loading of CD1d, we observed a marked dependence on detergent for loading of KRN7000 but not for C20:2 (Fig. 6*A*). This result suggested a significant difference in requirement for cofactors, such as acidic pH or lipid transfer proteins, that facilitate lipid loading onto CD1d in endosomes (38–41). We assessed this hypothesis further by using glutaraldehyde fixation of $CD1d^+$ antigen-presenting cells, which blocks antigen uptake and recycling of CD1d between endosomes and the plasma membrane. Vα14i NKT cell recognition of KRN7000 was markedly reduced if lipid loading was done after fixation of RMA-S.mCD1d cells, whereas recognition of C20:2 was unimpaired (Fig. 6*B*).

Similar conclusions were drawn from experiments by using A20 cells transfected with either WT or cytoplasmic tail-deleted CD1d (Fig. 6*C*). The tail-deleted CD1d mutant lacks the intracellular tyrosine-based sorting motif required for internalization and endosomal localization of CD1d (19). As was the case with RMA-S.mCD1d, WT CD1d-transfected A20 cells presented KRN7000 more potently than C20:2. However, the tail-deleted mutant presented C20:2 with at least 20-fold greater efficiency than KRN7000. Together, these results point to the conclusion that the T_H2-skewing C20:2 analogue had substantially less dependence on endosomal loading for presentation by CD1d when compared with compounds that produced a more mixed response with strong IFN-γ production, such as KRN7000.

Discussion

This study details *in vitro* and *in vivo* consequences of activation of Vα14i NKT cells with C20:2, a diunsaturated *N*-acyl substituted analogue of the prototypical α-GalCer, KRN7000. The T_H2 cytokine bias observed with C20:2 is not unique: OCH and other shortened fully saturated lipids have been shown to have this effect (13, 42). C20:2 differs from these other compounds in two potentially important respects. First, the *in vitro* potency of C20:2 for stimulation of certain Vα14i NKT cell functions (e.g., proliferation and secretion of IL-4 and IL-2) approaches that of KRN7000, whereas OCH appears to be a much weaker Vα14i NKT cell agonist. Second, staining with C20:2-loaded CD1d tetramers, as opposed to OCH, is undiminished compared with KRN7000. This finding would suggest that, as a therapeutic agent, C20:2 will be recognized by the identical global Vα14i NKT cell population (as KRN7000 is) and not limited to higher-affinity NKT cell subsets, as suggested for OCH (31).

A recent study showed that one mechanism by which OCH may induce a T_H2-biased cytokine response involves changes in IFN-γ production by Vα14i NKT cells themselves. Oki *et al.* (43) reported that the transcription factor gene *c-Rel*, a member of the NF-κB family of transcriptional regulators that is a crucial component of IFN-γ production, is inducibly transcribed in KRN7000-stimulated but not OCH-stimulated Vα14i NKT cells. Although we have not assessed *c-Rel* induction or other factors involved in IFN-γ production in response to C20:2, our findings did not suggest that early IFN-γ production by Vα14i NKT cells was different after activation with C20:2 versus KRN7000. Both lipids induced identical single-cell IFN-γ staining in Vα14i NKT cells and serum IFN-γ levels at 2 h after injection. However, in contrast to the apparent similarity in Vα14i NKT cells, NK cell IFN-γ production was significantly reduced and less sustained after *in vivo* administration of C20:2 compared with KRN7000. Hence, failure of C20:2 to fully activate downstream events leading to optimal NK cell secondary stimulation by activated Vα14i NKT cells appears to be the most likely mechanism by which C20:2 induces reduced IFN-γ and an apparent T_H2-biased systemic response.

C20:2 administration resulted also in a more rapid but less sustained CD69 up-regulation in NK and B cells, as well as a lack of a substantial Vα14i NKT cell expansion. These findings were surprising, given that TCR down-modulation observed on Vα14i NKT cells within the first few hours after C20:2 stimulation was similar to or greater than that induced by KRN7000 (Fig. 4*A* and data not shown), indicating strong TCR signaling in response to the analogue. These features of the response to C20:2 may be a further reflection of the failure of C20:2 to induce a full range of downstream events after Vα14i NKT cell activation, including the production of cytokines or other factors required to support the expansion of Vα14i NKT cells.

What mechanism can then be invoked to account for the altered cytokine response to C20:2 and other *N*-acyl variants of KRN7000? One intriguing possibility is provided by our analysis of requirements for presentation of C20:2 compared with KRN7000, which revealed marked differences between these glycolipids in their need for endosomal loading onto CD1d. CD1d and other CD1 proteins undergo transport into the endocytic pathway, leading to intracellular loading with lipid antigens and subsequent recycling to the cell surface (39). The importance of endosomal loading for KRN7000 most likely reflects the impact of factors in these compartments that facilitate the insertion of lipids into the CD1d ligand-binding groove. These factors include the acidic pH of the endosomal environment, as well as lipid transport proteins, such as saposins and GM2 activator protein (38, 40, 41). Our findings indicate that C20:2 can efficiently load onto CD1d in the absence of these endosomal cofactors. Consequently, we speculate that C20:2 may be strongly presented by any cell type that expresses surface CD1d, regardless of its ability to efficiently endocytose lipids from the extracellular space. This more widespread presentation could lead to a more pronounced presentation of C20:2 by nonprofessional antigen-presenting cell types compared with KRN7000. Because many cell types express CD1d, including all hematopoietic lineages and various types of epithelia (44–48), presentation of C20:2 by nonprofessional antigen-presenting cells may explain the more rapid trans-activation of bystander cells observed with C20:2. An alternative hypothesis is that the endosomal loading requirements of KRN7000 result in its preferential localization into CD1d molecules contained in membrane lipid rafts, whereas the permissive loading properties of

C20:2 would result in a more uniform glycolipid distribution across the cell membrane. Evidence of lipid raft localization of CD1d and raft influence on the T_H-bias of MHC class II-restricted $CD4^+$ T cells lend support to this model (49, 50). Either scenario would be expected to result in decreased delivery of costimulatory signals associated with professional antigen-presenting cells (e.g., dendritic cells) and, thus, lead to quantitative and qualitative differences in the outcome of Vα14i NKT cell stimulation. Consistent with both models, Vα14i NKT cell activation with KRN7000 *in vitro* in the presence of costimulatory blockade (anti-CD86) can polarize cytokine production to a T_H2 profile (22).

We have shown that structurally modified forms of α-GalCer with alterations in their *N*-acyl substituents can be designed to generate potent immunomodulators that stimulate qualitatively altered responses from Vα14i NKT cells. Our results confirm and extend several basic observations and principles established from earlier studies on less potent agonists, such as OCH. Further study of these and similar analogues may yield compounds with clear advantages for treatment or prevention of specific immunologic disorders or for the stimulation of protective host immunity against particular pathogens.

We thank R. Koganty and S. Gandhi (Biomira) for sharing their panel of synthetic glycosylceramides, which included compound BF1508-84; M. Kronenberg for the recombinant baculovirus used for production of soluble mouse CD1d; M. Taniguchi, T. Nakayama, A. Bendelac, M. Exley, S. Balk, S. Behar, and M. Kronenberg for gifts of mice and cell lines; Z. Hu for expert technical assistance; and T. DiLorenzo for critical reading of this manuscript. This work was supported by National Institutes of Health Grants AI45889, AI48933, and DK068690 (to S.A.P.), the Japan Human Sciences Foundation (T.Y. and S.A.P.), the Pharmaceutical and Medical Devices Agency (T.Y.), Medical Research Council Grants G9901077 and G0000895 (to G.S.B.), and Wellcome Trust Grants 060750 and 072021 (to G.S.B.). G.S.B. is a Lister Jenner Research Fellow.

1. Lantz, O. & Bendelac, A. (1994) *J. Exp. Med.* **180**, 1097–1106.
2. Brossay, L. & Kronenberg, M. (1999) *Immunogenetics* **50**, 146–151.
3. Godfrey, D. I., Hammond, K. J., Poulton, L. D., Smyth, M. J. & Baxter, A. G. (2000) *Immunol. Today* **21**, 573–583.
4. Wilson, S. B. & Delovitch, T. L. (2003) *Nat. Rev. Immunol.* **3**, 211–222.
5. Poulton, L. D., Smyth, M. J., Hawke, C. G., Silveira, P., Shepherd, D., Naidenko, O. V., Godfrey, D. I. & Baxter, A. G. (2001) *Int. Immunol.* **13**, 887–896.
6. Gombert, J.-M., Tancrede-Bohin, T., Hameg, A., do Carmo Leite-de-Moraes, M., Vicari, A. P., Bach, J.-F. & Herbelin, A. (1996) *Int. Immunol.* **8**, 1751–1758.
7. Van Der Vliet, H. J., Von Blomberg, B. M., Nishi, N., Reijm, M., Voskuyl, A. E., van Bodegraven, A. A., Polman, C. H., Rustemeyer, T., Lips, P., Van Den Eertwegh, A. J., *et al.* (2001) *Clin. Immunol.* **100**, 144–148.
8. Taniguchi, M., Harada, M., Kojo, S., Nakayama, T. & Wakao, H. (2003) *Annu. Rev. Immunol.* **21**, 483–513.
9. Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Motoki, K., Ueno, H., Nakagawa, R., Sato, H., Kondo, E., *et al.* (1997) *Science* **278**, 1626–1629.
10. Wang, B., Geng, Y. B. & Wang, C. R. (2001) *J. Exp. Med.* **194**, 313–320.
11. Sharif, S., Arreaza, G. A., Zucker, P., Mi, Q. S., Sondhi, J., Naidenko, O. V., Kronenberg, M., Koezuka, Y., Delovitch, T. L., Gombert, J. M., *et al.* (2001) *Nat. Med.* **7**, 1057–1062.
12. Hong, S., Wilson, M. T., Serizawa, I., Wu, L., Singh, N., Naidenko, O. V., Miura, T., Haba, T., Scherer, D. C., Wei, J., *et al.* (2001) *Nat. Med.* **7**, 1052–1056.
13. Miyamoto, K., Miyake, S. & Yamamura, T. (2001) *Nature* **413**, 531–534.
14. Mizuno, M., Masumura, M., Tomi, C., Chiba, A., Oki, S., Yamamura, T. & Miyake, S. (2004) *J. Autoimmun.* **23**, 293–300.
15. Sonoda, K. H., Exley, M., Snapper, S., Balk, S. P. & Stein-Streilein, J. (1999) *J. Exp. Med.* **190**, 1215–1226.
16. Cui, J., Shin, T., Kawano, T., Sato, H., Kondo, E., Toura, I., Kaneko, Y., Koseki, H., Kanno, M. & Taniguchi, M. (1997) *Science* **278**, 1623–1626.
17. Behar, S. M., Podrebarac, T. A., Roy, C. J., Wang, C. R. & Brenner, M. B. (1999) *J. Immunol.* **162**, 161–167.
18. Brossay, L., Tangri, S., Bix, M., Cardell, S., Locksley, R. & Kronenberg, M. (1998) *J. Immunol.* **160**, 3681–3688.
19. Prigozy, T. I., Naidenko, O., Qasba, P., Elewaut, D., Brossay, L., Khurana, A., Natori, T., Koezuka, Y., Kulkarni, A. & Kronenberg, M. (2001) *Science* **291**, 664–667.
20. Porcelli, S., Morita, C. T. & Brenner, M. B. (1992) *Nature* **360**, 593–597.
21. Matsuda, J. L., Naidenko, O. V., Gapin, L., Nakayama, T., Taniguchi, M., Wang, C. R., Koezuka, Y. & Kronenberg, M. (2000) *J. Exp. Med.* **192**, 741–754.
22. Pal, E., Tabira, T., Kawano, T., Taniguchi, M., Miyake, S. & Yamamura, T. (2001) *J. Immunol.* **166**, 662–668.
23. Matsuda, J. L., Gapin, L., Baron, J. L., Sidobre, S., Stetson, D. B., Mohrs, M., Locksley, R. M. & Kronenberg, M. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 8395–8400.
24. Crowe, N. Y., Uldrich, A. P., Kyparissoudis, K., Hammond, K. J., Hayakawa, Y., Sidobre, S., Keating, R., Kronenberg, M., Smyth, M. J. & Godfrey, D. I. (2003) *J. Immunol.* **171**, 4020–4027.
25. Wilson, M. T., Johansson, C., Olivares-Villagomez, D., Singh, A. K., Stanic, A. K., Wang, C. R., Joyce, S., Wick, M. J. & Van Kaer, L. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 10913–10918.
26. Carnaud, C., Lee, D., Donnars, O., Park, S. H., Beavis, A., Koezuka, Y. & Bendelac, A. (1999) *J. Immunol.* **163**, 4647–4650.
27. Schmieg, J., Yang, G., Franck, R. W. & Tsuji, M. (2003) *J. Exp. Med.* **198**, 1631–1641.
28. Hayakawa, Y., Takeda, K., Yagita, H., Kakuta, S., Iwakura, Y., Van Kaer, L., Saiki, I. & Okumura, K. (2001) *Eur. J. Immunol.* **31**, 1720–1727.
29. Ebert, G. & MacDonald, H. R. (2000) *Eur. J. Immunol.* **30**, 985–992.
30. Kitamura, H., Ohta, A., Sekimoto, M., Sato, M., Iwakabe, K., Nakui, M., Yahata, T., Meng, H., Koda, T., Nishimura, S., *et al.* (2000) *Cell. Immunol.* **199**, 37–42.
31. Stanic, A. K., Shashidharamurthy, R., Bezbradica, J. S., Matsuki, N., Yoshimura, Y., Miyake, S., Choi, E. Y., Schell, T. D., Van Kaer, L., Tevethia, S. S., *et al.* (2003) *J. Immunol.* **171**, 4539–4551.
32. Benlagha, K., Kyin, T., Beavis, A., Teyton, L. & Bendelac, A. (2002) *Science* **296**, 553–555.
33. Gumperz, J. E., Miyake, S., Yamamura, T. & Brenner, M. B. (2002) *J. Exp. Med.* **195**, 625–636.
34. Lee, P. T., Benlagha, K., Teyton, L. & Bendelac, A. (2002) *J. Exp. Med.* **195**, 637–641.
35. Gadue, P. & Stein, P. L. (2002) *J. Immunol.* **169**, 2397–2406.
36. Pellicci, D. G., Hammond, K. J., Uldrich, A. P., Baxter, A. G., Smyth, M. J. & Godfrey, D. I. (2002) *J. Exp. Med.* **195**, 835–844.
37. Sidobre, S., Naidenko, O. V., Sim, B. C., Gascoigne, N. R., Garcia, K. C. & Kronenberg, M. (2002) *J. Immunol.* **169**, 1340–1348.
38. Kang, S. J. & Cresswell, P. (2004) *Nat. Immunol.* **5**, 175–181.
39. Moody, D. B. & Porcelli, S. A. (2003) *Nat. Rev. Immunol.* **3**, 11–22.
40. Winau, F., Schwierzeck, V., Hurwitz, R., Remmel, N., Sieling, P. A., Modlin, R. L., Porcelli, S. A., Brinkmann, V., Sugita, M., Sandhoff, K., *et al.* (2004) *Nat. Immunol.* **5**, 169–174.
41. Zhou, D., Cantu, C., III, Sagiv, Y., Schrantz, N., Kulkarni, A. B., Qi, X., Mahuran, D. J., Morales, C. R., Grabowski, G. A., Benlagha, K., *et al.* (2004) *Science* **303**, 523–527.
42. Goff, R. D., Gao, Y., Mattner, J., Zhou, D., Yin, N., Cantu, C., III, Teyton, L., Bendelac, A. & Savage, P. B. (2004) *J. Am. Chem. Soc.* **126**, 13602–13603.
43. Oki, S., Chiba, A., Yamamura, T. & Miyake, S. (2004) *J. Clin. Invest.* **113**, 1631–1640.
44. Bonish, B., Jullien, D., Dutronc, Y., Huang, B. B., Modlin, R., Spada, F. M., Porcelli, S. A. & Nickoloff, B. J. (2000) *J. Immunol.* **165**, 4076–4085.
45. Colgan, S. P., Pitman, R. S., Nagaishi, T., Mizoguchi, A., Mizoguchi, E., Mayer, L. F., Shao, L., Sartor, R. B., Subjeck, J. R. & Blumberg, R. S. (2003) *J. Clin. Invest.* **112**, 745–754.
46. Brossay, L., Jullien, D., Cardell, S., Sydora, B. C., Burdin, N., Modlin, R. L. & Kronenberg, M. (1997) *J. Immunol.* **159**, 1216–1224.
47. Park, S. H., Roark, J. H. & Bendelac, A. (1998) *J. Immunol.* **160**, 3128–3134.
48. Roark, J. H., Park, S. H., Jayawardena, J., Kavita, U., Shannon, M. & Bendelac, A. (1998) *J. Immunol.* **160**, 3121–3127.
49. Lang, G. A., Maltsev, S. D., Besra, G. S. & Lang, M. L. (2004) *Immunology* **112**, 386–396.
50. Buatois, V., Baillet, M., Becart, S., Mooney, N., Leserman, L. & Machy, P. (2003) *J. Immunol.* **171**, 5812–5819.

IFN-γ–mediated negative feedback regulation of NKT-cell function by CD94/NKG2

Tsuyoshi Ota, Kazuyoshi Takeda, Hisaya Akiba, Yoshihiro Hayakawa, Kouetsu Ogasawara, Yoshinori Ikarashi, Sachiko Miyake, Hiro Wakasugi, Takashi Yamamura, Mitchell Kronenberg, David H. Raulet, Katsuyuki Kinoshita, Hideo Yagita, Mark J. Smyth, and Ko Okumura

Activation of invariant natural killer T (iNKT) cells with CD1d-restricted T-cell receptor (TCR) ligands is a powerful means to modulate various immune responses. However, the iNKT-cell response is of limited duration and iNKT cells appear refractory to secondary stimulation. Here we show that the CD94/NKG2A inhibitory receptor plays a critical role in down-regulating iNKT-cell responses. Both TCR and NK-cell receptors expressed by iNKT cells were rapidly down-modulated by priming with α-galactosylceramide (α-GalCer) or its analog OCH [(2*S*,3*S*,4*R*)-1-*O*-(α-D-galactopyranosyl)-*N*-tetracosanoyl-2-amino-1,3,4-nonanetriol)]. TCR and CD28 were re-expressed more rapidly than the inhibitory NK-cell receptors CD94/NKG2A and Ly49, temporally rendering the primed iNKT cells hyperreactive to ligand restimulation. Of interest, α-GalCer was inferior to OCH in priming iNKT cells for subsequent restimulation because α-GalCer–induced interferon γ (IFN-γ) up-regulated Qa-1^b expression and Qa-1^b in turn inhibited iNKT-cell activity via its interaction with the inhibitory CD94/NKG2A receptor. Blockade of the CD94/NKG2–Qa-1^b interaction markedly augmented recall and primary responses of iNKT cells. This is the first report to show the critical role for NK-cell receptors in controlling iNKT-cell responses and provides a novel strategy to augment the therapeutic effect of iNKT cells by priming with OCH or blocking of the CD94/NKG2A inhibitory pathway in clinical applications. (Blood. 2005;106:184-192)

Introduction

Natural killer T (NKT) cells are a special T-cell population coexpressing the T-cell receptor (TCR) and NK-cell receptors such as NK1.1.[1-4] Invariant NKT (iNKT) cells express a Vα14Jα18 chain (Vα24Jα15 in humans) and a semivariant TCR β-chain that is largely biased toward Vβ8.2 (Vβ11 in humans), Vβ2, and Vβ7.[1-4] This TCR recognizes glycolipid antigens, such as α-galactosylceramide (α-GalCer), its analogs including OCH (a sphingosine-truncated analog of α-GalCer; (2*S*,3*S*,4*R*)-1-*O*-(α-D-galactopyranosyl)-*N*-tetracosanoyl-2-amino-1,3,4-nonanetriol), and isoglobotrihexosylceramide (iGb3), presented on the major histocompatibility complex (MHC) class I–like molecule CD1d.[1-10] In addition, costimulatory signals, mediated through antigen-presenting cells that express CD80/86 interacting with CD28 expressed by iNKT cells, critically regulate iNKT-cell activation in a similar manner to conventional T cells.[11,12] One of most striking characteristics of iNKT cells is their ability to promptly secrete various cytokines, including both interferon γ (IFN-γ) and interleukin 4 (IL-4), after their encounter with antigens (Ag's),[1-4] which is reminiscent of effector/memory T cells. Accordingly, iNKT cells are thought to be potent immunoregulatory cells and their activation by ligands, such as α-GalCer and OCH, has been shown to be a powerful means to modulate various immune responses, including protective immunity, autoimmunity, and antitumor immunity.[1-4,6,9,11,13,14]

Despite an accumulation of studies concerning iNKT-cell activation of bystander immune cells, relatively little is known about the fate of Ag-primed iNKT cells themselves. Previous studies have shown that iNKT cells disappear quickly after their activation following TCR ligation or IL-12 stimulation.[15-17] This phenomenon was initially attributed to increased activation-induced cell death (AICD) of iNKT cells, consistent with repopulation and homeostatic proliferation of peripheral iNKT cells after their rapid recruitment from the bone marrow.[15] More recently, however, several studies have reported that down-regulation of TCR and NK1.1 cell surface expression on iNKT cells is the primary reason for the apparent disappearance of iNKT cells following α-GalCer treatment.[18-20] Indeed a substantial iNKT-cell proliferation was observed in peripheral lymphoid organs following

From the Departments of Immunology, and Obstetrics and Gynecology, Juntendo University School of Medicine, Tokyo, Japan; Cancer Immunology Program, Sir Donald and Lady Trescowthick Laboratories, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia; Department of Microbiology and Immunology and the Cancer Research Institute, University of California San Francisco, San Francisco, CA; Pharmacology Division, National Cancer Center Research Institute, Tokyo, Japan; Department of Immunology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan; Division of Developmental Immunology, La Jolla Institute for Allergy and Immunology, San Diego, CA; and Department of Molecular and Cell Biology and Cancer Research Laboratory, University of California, Berkeley, CA.

Submitted November 8, 2004; accepted February 27, 2005. Prepublished online as *Blood* First Edition Paper, March 3, 2005; DOI 10.1182/blood-2004-11-4257.

Supported by research grants from the Human Frontier Science Program Organization; the Organization for Pharmaceutical Safety and Research; the Ministry of Education, Science, and Culture, Japan; the National Health and Medical Research Council of Australia; and the National Institutes of Health.

T.O. and K.T. contributed equally to this work.

Reprints: Kazuyoshi Takeda, Department of Immunology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan; e-mail: ktakeda@med.juntendo.ac.jp.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

α-GalCer treatment.[18-20] These findings suggested an interesting possibility that Ag-primed iNKT cells may develop an effector/memory subpopulation, which exerts even more potent function than unprimed iNKT cells. Here, we now demonstrate that priming of iNKT cells with their TCR ligands induces a dynamic modulation of TCR, NK-cell receptors (NK1.1, CD94/NKG2, NKG2D, and Ly49), and a costimulatory receptor (CD28). Differential kinetics of re-expression of these molecules on the cell surface temporally renders the primed iNKT cells hyperresponsive to secondary Ag stimulation. Of importance, the recall response of α-GalCer–primed iNKT cells is strictly regulated by an IFN-γ–dependent negative feedback mechanism where IFN-γ upregulates Qa-1^b expression and subsequent ligation of CD94/NKG2 inhibits iNKT-cell activity. Although Qa-1^b can interact with NKG2A, NKG2C, and NKG2E, it has been previously reported that the majority of CD94/NKG2 expressed in mice is the inhibitory CD94/NKG2A receptor and that the Qa-1^b–CD94/NKG2A interaction plays a critical role in negative regulation of NK-cell responses to self.[21-23] We confirm that iNKT cells preferentially express CD94/NKG2A and hence blockade of the Qa-1^b–NKG2A interaction markedly augmented recall responses of primed iNKT cells and primary responses of naive iNKT cells to α-GalCer. These findings are an important step to improve the efficacy of iNKT-cell–targeting therapeutics against tumor, infection, and autoimmune diseases since they demonstrate a means to modulate the adjuvant nature of iNKT cells by combined treatment with iNKT-cell glycolipid ligands and antagonistic monoclonal antibodies (mAbs).

Materials and methods

Mice

Wild-type (WT) C57BL/6 (B6) mice were obtained from Charles River Japan (Yokohama, Japan). IFN-γ–deficient (IFN-$\gamma^{-/-}$) B6 mice were kindly provided by Y. Iwakura (University of Tokyo).[24] All mice were maintained under specific pathogen-free conditions and used in accordance with the institutional guidelines of Juntendo University.

Reagents

A synthetic form of α-GalCer was obtained from Kirin Brewery (Gunma, Japan) and OCH was derived as described previously.[6] In most experiments, mice were intraperitoneally injected with 2 μg of α-GalCer or OCH in 200 μL of phosphate-buffered saline (PBS) for priming and boosting. Dimethyl sulfoxide (DMSO; 0.1%) was used as the vehicle control. Phycoerythrin (PE)–conjugated tetrameric CD1d molecules loaded with α-GalCer (α-GalCer/CD1d) were prepared as described.[17] The anti-NKG2A/C/E (NKG2) mAb, 20d5, and the anti-NKG2D mAb (CX5) were generated as described previously.[21,25] Fab fragments of 20d5 and anti–Qa-1^b mAb (BD Pharmingen, San Diego, CA) were prepared using the Fab preparation kit (Pierce, Rockford, IL) as described.[26]

Flow cytometric analysis

Mononuclear cells (MNCs) were prepared from spleen and liver as described.[11] Cells were first preincubated with antimouse CD16/32 (2.4G2) mAb to avoid nonspecific binding of mAbs to FcγR. Surface and intracellular expression of molecules by iNKT cells were analyzed on electronically gated α-GalCer/CD1d tetramer$^+$ cells on 4-color flow cytometry using a FACSCaliber (BD Bioscience, San Jose, CA). Intracellular staining was performed with a BD Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer's instructions. Intracellular TCR in NKT cells was detected with a mixture of PE-conjugated antimouse Vβ2 TCR mAb (B20.6), anti-Vβ7 TCR mAb (TR310), and anti-Vβ8 TCR mAb (F23.1), or α-GalCer–loaded recombinant soluble dimeric mouse CD1d:immunoglobulin (CD1d:Ig; BD Pharmingen) and PE-conjugated anti-mouse IgG1 mAb (A85-1; BD Pharmingen). Surface and intracellular molecules were analyzed on electronically gated intracellular Vβ2/7/8$^+$ cells 1 day after α-GalCer or OCH injection. Surface and intracellular molecules were stained with fluorescein isothiocyanate (FITC)– or allophycocyanin (APC)–conjugated NK1.1 mAb (PK136); FITC- or biotin-conjugated antimouse CD94 mAb (18d3); FITC- or biotin-conjugated antimouse NKG2A/C/E (NKG2) mAb (20d5); biotin-conjugated antimouse NKG2A^{B6} (16a11); FITC-conjugated antimouse Ly49 mAbs (anti-Ly49D mAb [4E5] or a mixture of anti-Ly49A^{B6} [A1], anti-Ly49C/I mAb [5E6], anti-Ly49G2 mAb [4D11], and antimouse Ly49I mAb [YL1-90]); biotin-conjugated anti-NKG2D mAb (CX5); APC- or biotin-conjugated anti-mouse CD28 mAb (37.51); biotin-conjugated antimouse Qa-1^b mAb (6A8.6F10.1A6); FITC-, PE-, PE-cyanin 5 (PE-Cy5)–, APC-, or biotin-conjugated isotype-matched control mAbs (G155-178, MOPC-31C, R35-95, A95-1, R3-34, and Ha4/8); and Cy5-conjugated streptavidin. All of these reagents were purchased from BD Pharmingen, except for antimouse CD94 mAb, anti-NKG2A^{B6} mAb, anti-NKG2D mAb, and anti-CD28 mAb from eBioscience (San Diego, CA).

Cell preparation and in vitro stimulation

Freshly isolated splenic MNCs from vehicle-, α-GalCer–, or OCH-primed mice (5×10^5) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, and 25 mM $NaHCO_3$ in humidified 5% CO_2 at 37°C in 96-well U-bottom plates (Corning, Corning, NY) as previously described.[11] Cells were stimulated with 100 ng/mL α-GalCer, OCH, or vehicle (0.1% DMSO) in the presence or absence of 10-μg/mL Fab fragments of isotype-matched control mAbs, anti-NKG2 mAb, or antimouse Qa-1^b mAb, or intact antimouse CD80 (16-10A1) and antimouse CD86 (PO 3.1) mAbs (eBioscience). After 24 to 48 hours, cell-free culture supernatants were harvested to determine IFN-γ and IL-4 levels by enzyme-linked immunosorbent assay (ELISA).

Coculture of iNKT cells and DCs

Freshly isolated hepatic MNCs were stained with PE-conjugated α-GalCer/CD1d tetramer, and positive cells were enriched by autoMACS using anti-PE microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Enriched iNKT cells were then sorted on a FACS Vantage (BD Bioscience) to obtain highly purified (98%-99%) iNKT cells. Splenic dendritic cells (DCs) were prepared according to the reported method.[27,28] Purified iNKT cells (10^5) and DCs (5×10^4) were cocultured as previously described[11,29,30] with 100 ng/mL α-GalCer or vehicle (0.1% DMSO) in the presence or absence of 10-μg/mL Fab fragments of isotype-matched control mAbs, anti-NKG2A mAb, or anti–Qa-1^b mAb. After 24 to 72 hours, cell-free culture supernatants were harvested to determine IFN-γ and IL-4 levels by ELISA.

ELISA

IFN-γ and IL-4 levels in the culture supernatants or the sera were determined by using mouse IFN-γ– or IL-4–specific ELISA kits (OptEIA; BD Bioscience Pharmingen) according to the manufacturer's instructions.

Cytotoxicity assay

Cytotoxic activity was tested against NK-cell–sensitive YAC-1 cells and NK-cell–resistant P815 cells by a standard 4-hour ^{51}Cr-release assay as previously described.[11] Effector cells (hepatic and splenic MNCs) were prepared from mice 24 hours after the last intraperitoneal injection of α-GalCer, OCH, or vehicle. Some mice were administered with 300 μg of isotype-matched control Ig or anti-NKG2 mAb intraperitoneally 2 days before the last α-GalCer injection. Specific cytotoxicity was calculated as previously described.[11]

Experimental lung metastases

B6 mice were intraperitoneally injected with OCH, α-GalCer, or vehicle, and then intravenously inoculated with B16 melanoma cells (1×10^5,