cytokine production, cytotoxic activity, or the antimetastatic effect caused by the α-GalCer injection. These results indicated that the iNKT-cell activation by α-GalCer was limited by CD94/NKG2-mediated suppression and blockade of CD94/NKG2 could significantly improve the antitumor effect of a secondary α-GalCer treatment.

Discussion

In this study, we have shown that activation of iNKT cells is critically regulated by CD94/NKG2. In addition to TCR and CD28, naive iNKT cells express activating (NK1.1, NKG2D, and Ly49D) or inhibitory (CD94/NKG2 and Ly49A/C/I/G2) NK-cell receptors. All of these cell surface receptors were rapidly down-modulated upon priming of iNKT cells with their TCR ligands (α-GalCer or OCH). Two to 3 days after the priming, iNKT cells re-expressed TCR and CD28 on their surface, but CD94/NKG2 and Ly49 remained down-modulated. This pattern of expression enabled the primed iNKT cells to produce a larger amount of cytokines upon secondary stimulation with α-GalCer. Of interest, OCH was superior to α-GalCer in priming iNKT cells for a secondary response to α-GalCer, resulting in a markedly enhanced antimetastatic effect. We found that IFN-γ induced by α-GalCer priming up-regulated Qa-1^b, which in turn suppressed the secondary iNKT-cell activation via CD94/NKG2. Thus, the blockade of CD94/NKG2 markedly enhanced the antimetastatic effect of α-GalCer after α-GalCer priming. These findings revealed a negative feedback regulation of iNKT-cell activation by IFN-γ–inducible Qa-1^b and provided a novel strategy to improve the antimetastatic effect of α-GalCer by priming with OCH or by blocking CD94/NKG2-mediated suppression.

It was unexpected that OCH was far more effective than α-GalCer in priming the secondary iNKT-cell responses to α-GalCer, since OCH and α-GalCer similarly modulated iNKT-cell surface receptors and OCH was rather inferior to α-GalCer in expanding iNKT cells upon priming (data not shown) as recently reported.[6,9] We hypothesized that IFN-γ produced by iNKT cells upon priming with α-GalCer might be responsible for this difference, since OCH did not induce IFN-γ production in vivo. We found that α-GalCer priming up-regulated the expression of Qa-1^b in an IFN-γ–dependent manner, which suppressed iNKT-cell activation in response to secondary α-GalCer stimulation in vitro and in vivo. Qa-1^b is an MHC class Ib molecule broadly expressed on leukocytes and it predominantly presents a canonical signal peptide of classical MHC class Ia molecules, called Qa-1 determinant modifier (Qdm), in a transporter associated with antigen presentation (TAP)–dependent manner, thereby indirectly representing cellular MHC class Ia levels.[32,33] The up-regulation of Qa-1^b expression by IFN-γ might be due to increased transcription of *Qa-1^b* and/or increased TAP-mediated loading of Qdm onto Qa-1^b. The Qdm/Qa-1^b complex is recognized by inhibitory CD94/NKG2A and activating CD94/NKG2C or E receptors.[21-23] The CD94/NKG2 receptors expressed on naive and primed iNKT cells were predominantly CD94/NKG2A as estimated by staining with an NKG2A-specific mAb as previously reported.[22] It has been shown that CD94/NKG2A expressed on NK cells and $CD8^+$ T cells suppressed their activation.[34] However, our present results are the first indication that iNKT-cell activation is critically regulated by CD94/NKG2A. Similarly, inhibitory Ly49 receptors, which recognize MHC class Ia molecules directly, have been reported to suppress α-GalCer–induced iNKT-cell activation.[30,35] Since CD94/NKG2A is more frequently expressed on iNKT cells than Ly49, it may play a more dominant role in regulating iNKT-cell activation. The IFN-γ–mediated Qa-1^b up-regulation may be a negative feedback mechanism to maintain self-tolerance of iNKT cells and avoid a pathogenic effect of iNKT cells.[36-39] It will be interesting to explore the iNKT-cell functions in Qa-1^b–deficient[40] or CD94-deficient[22] mice in future studies.

Consistent with recent reports,[18-20] we observed a rapid down-modulation of TCR and NK1.1 on the surface of iNKT cells upon priming with their TCR ligands, which mostly accounted for the apparent disappearance of iNKT cells. However, intracellular staining 1 day after α-GalCer priming demonstrated a 20% to 30% reduction of iNKT-cell numbers compared with untreated mice (data not shown), and some annexin V–positive iNKT cells were detected in the liver and spleen promptly after α-GalCer injection as we reported previously.[16] Therefore, some minor fraction of iNKT cells appeared to be susceptible to AICD upon α-GalCer priming. A significant increase of CD94/$NKG2^+$ iNKT cells after α-GalCer priming suggested that these cells were more resistant to AICD than CD94/$NKG2^-$ iNKT cells. This preferential survival and/or expansion of CD94/$NKG2^+$ iNKT cells might be at least partly responsible for the higher sensitivity of primed iNKT cells to Qa-1^b and CD94/NKG2-mediated suppression.

We and others have recently shown that the expansion of iNKT cells is maximal 3 days after α-GalCer priming and then iNKT-cell numbers gradually return to normal levels by homeostatic mechanisms within 7 to 10 days.[9,18-20] This is consistent with the kinetics of recall responses of α-GalCer– or OCH-primed mice to α-GalCer, suggesting that the enhanced secondary responses were mainly due to expansion of iNKT cells after priming. However, the enhanced secondary responses were mostly maintained up to 10 days after α-GalCer priming if CD94/NKG2-mediated suppression was blocked at the secondary α-GalCer stimulation. This suggests that the primed iNKT cells with a high capacity to produce cytokines upon restimulation persist (typical of effector/ memory T cells), although they are under a strict regulation by CD94/NKG2A-mediated suppression.

A recent study has shown that the recognition of α-GalCer analogues was influenced by the TCR Vβ repertoires of iNKT cells. OCH was preferentially recognized by $V\beta8^+$ iNKT cells, which also have a higher avidity for α-GalCer than $V\beta7^+$ iNKT cells.[7,41] Thus, a preferential expansion of $V\beta8^+$ iNKT cells after OCH or α-GalCer priming might also be responsible for the enhanced responses of primed iNKT cells to α-GalCer restimulation in vitro and in vivo.

We have previously shown that α-GalCer administration into naive mice induces sustained IFN-γ production and cytotoxic activity, which were mediated by NK cells secondarily activated by IFN-γ derived from iNKT cells and IL-12 derived from DCs.[16,29,31] Thus, depletion of NK cells mostly abrogated the sustained α-GalCer response and consequently impaired the antimetastatic effect of α-GalCer. In contrast, α-GalCer administration into OCH-primed mice induced a greatly enhanced IFN-γ production at 5 hours but not at 16 to 20 hours, which was not reduced by NK-cell depletion (data not shown). This indicated that OCH priming mainly enhanced IFN-γ production by iNKT cells themselves, rather that secondary activated NK cells, upon the secondary α-GalCer stimulation. However, the markedly enhanced cytotoxic activity of liver and spleen MNCs 24 hours after α-GalCer boost in OCH-primed mice was mostly abrogated by NK-cell depletion (K.T., unpublished data, May 2004). In addition, the

significantly augmented antimetastatic effect of α-GalCer in OCH-primed mice was significantly inhibited by NK-cell depletion (K.T., unpublished data, May 2004). These data suggested that IFN-γ–activated NK cells were mainly responsible for the antimetastatic effect of α-GalCer in the OCH-primed mice. In this context, the CD94/NKG2 blockade might augment the antimetastatic effect of α-GalCer by enhancing the activation of not only iNKT cells but also NK cells, since NK cells also express CD94/NKG2A inhibitory receptors. Thus, blockade of the CD94/NKG2A suppressive pathway may be effective at either the induction or effector phase of the α-GalCer–induced antitumor effect.

The most notable finding of this study is that the antimetastatic effect of α-GalCer was greatly improved by the OCH-priming or the CD94/NKG2A blockade. OCH was a weak inducer of iNKT-cell expansion and IL-4 production but did not induce IFN-γ production or antimetastatic activity by itself.[6,7,9] However, OCH modulated iNKT-cell surface receptors as efficiently as α-GalCer. These OCH-primed iNKT cells produced a huge amount of IFN-γ upon secondary α-GalCer restimulation in vivo, resulting in a potent antimetastatic effect. The inability of OCH to induce IFN-γ was a beneficial property for priming secondary α-GalCer responses because IFN-γ down-regulated the secondary iNKT-cell responses by up-regulating Qa-1^b and thus CD94/NKG2A-mediated suppression. Recent studies have demonstrated quantitative and qualitative differences in the in vivo response of iNKT cells to distinct α-GalCer analogues, including OCH and β-GalCer.[9] Like OCH, in our preliminary experiments, priming with β-GalCer a26,[9] another weak iNKT-cell ligand inducing poor cytokine production, also potently enhanced iNKT-cell responses to α-GalCer restimulation (data not shown). α-GalCer and OCH have been shown to activate human Vα24 iNKT cells in a similar manner to murine Vα14 iNKT cells in vitro,[3,4] and α-GalCer is now in early clinical trials in cancer patients.[42,43] Therefore, priming with OCH may be a novel strategy to improve the therapeutic effect of α-GalCer in such patients. Further exploration of an α-GalCer analog with a better priming effect is also warranted. CD94/NKG2A blockade might be also applicable to improve the antitumor effect of α-GalCer in humans. In addition to an antitumor effect, α-GalCer has been shown to protect mice against infections and autoimmune diseases.[1-4,13,14] Therefore, the priming with OCH and the blockade of CD94/NKG2A may also be applicable to improve the therapeutic effect of α-GalCer in these diseases. However, it has also been shown that α-GalCer occasionally exacerbated autoimmune diseases, depending on the model and/or administration protocol.[44] Moreover, overactivation of iNKT cells can induce tissue pathologies.[36-39] Therefore, further studies are needed to determine the optimal prime/boost protocol or blockade of NK-cell receptors in iNKT-cell–targeting therapy for the safe treatment of tumor, infections, and autoimmune diseases.

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

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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 $J\alpha18^{-/-}$ 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-

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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.

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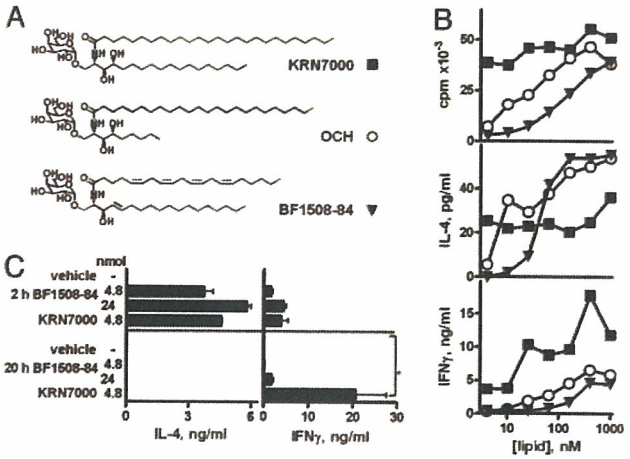

Fig. 1. Induction of a T_H2-polarized cytokine response by an unsaturated analogue of α-GalCer. (*A*) Glycolipid structures. (*B*) [^{3}H]thymidine incorporation and supernatant IL-4 and IFN-γ levels in 72-h splenocyte cultures with graded amounts of glycolipid. Means from triplicate cultures are shown; 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 ($\leq 10^6$) 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

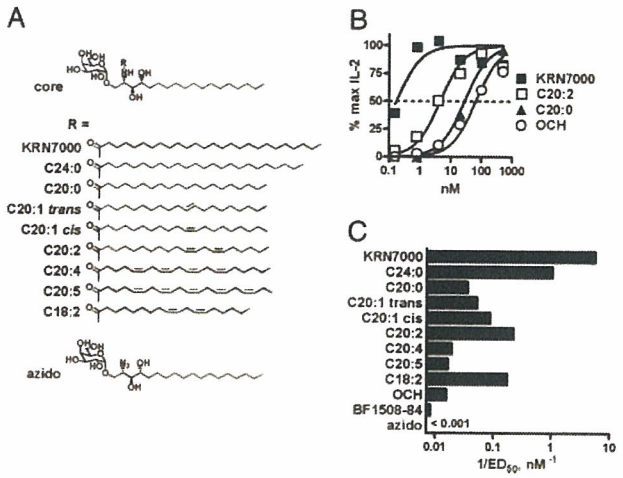

Fig. 2. Recognition of a panel of unsaturated analogues of KRN7000 by a canonical Vα14i NKT hybridoma. (*A*) Analogue structures. (*B*) Dose–response curves showing IL-2 production by hybridoma DN3A4–1.2 after stimulation with RMA-S.mCD1d cells pulsed with various doses of glycolipid. Maximal IL-2 concentrations 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.

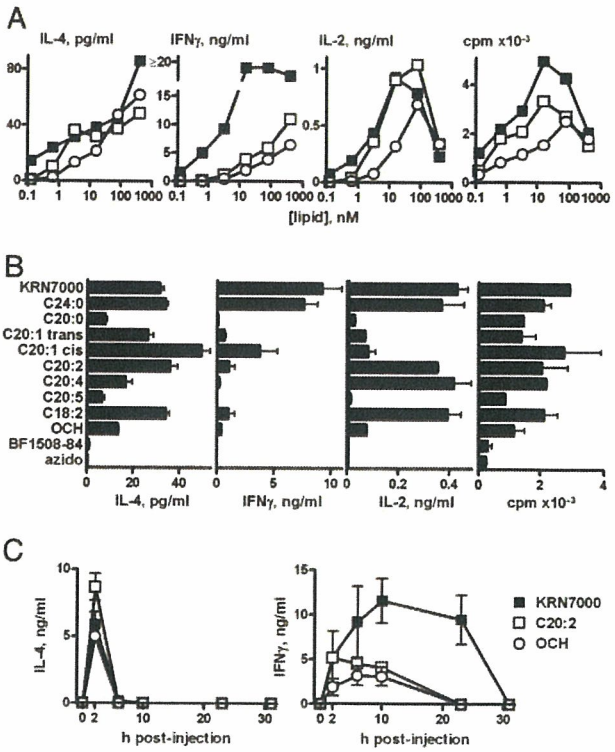

Fig. 3. T_H2-skewing of *in vitro* and *in vivo* cytokine responses to C20:2. (*A*) Dose–response curves reporting 48 h IL-4, IFN-γ, or IL-2 production, and cell proliferation of splenocytes in response to KRN7000, C20:2, and OCH. Means of duplicate cultures are shown; SEM were <10% of the means. (*B*) 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

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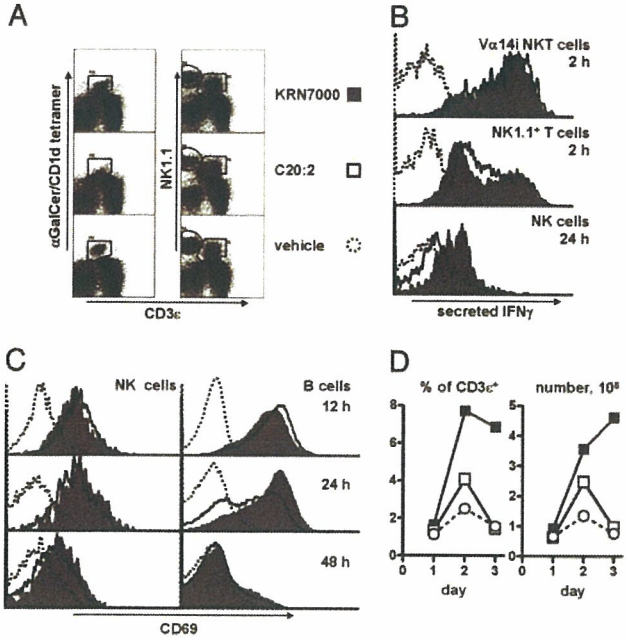

Fig. 4. Sequelae of KRN7000 and C20:2-induced Vα14i NKT cell activation. (*A*) Vα14i NKT cell (tetramer$^+$ CD3ε^{int}), NK cell (NK1.1$^+$ CD3ε^-), and NK1.1$^+$ T cell (NK1.1^{int} CD3ε^{int}) identification by FACS in splenocytes from mice given KRN7000, C20:2, or vehicle i.p. 2 h earlier. Lymphocytes 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.

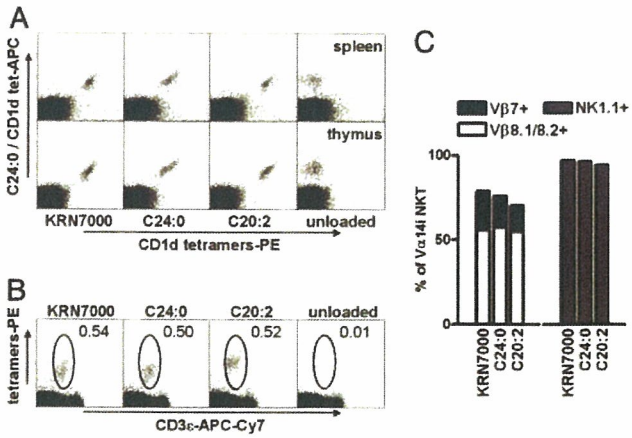

Fig. 5. Recognition of KRN7000, C24:0, and C20:2 by the same population of Vα14i NKT cells. (*A*) Costaining of C57BL/6 splenocytes or thymocytes with allophycocyanin-conjugated CD1d tetramers assembled with C24:0, and phycoerythrin-labeled CD1d tetramers assembled with various analogues. (*B*) Thymocytes 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.

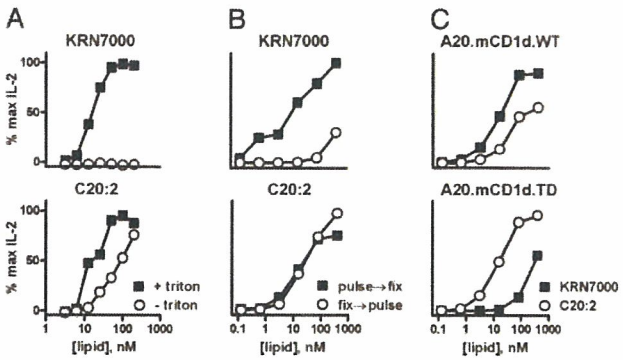

Fig. 6. Differential requirements for CD1d loading with KRN7000 and C20:2. IL-2 response of hybridoma DN3A4–1.2 to glycolipid presentation in three *in vitro* CD1d presentation systems: platebound CD1d loaded with varying amounts of KRN7000 or C20:2 in the presence or absence of the detergent Triton 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.

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＜特集Ⅰ　サイトカイン・ケモカインからみた多発性硬化症の病型と病態＞

DNAマイクロアレイによる多発性硬化症の免疫病態の解析

佐藤　準一[1,2)]

DNA Microarray Analysis Clarifies Immunopathogenesis of Multiple Sclerosis

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Abstract

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) white matter mediated by an autoimmune process whose development is triggered by a complex interplay of both genetic and environmental factors. MS shows a great range of phenotypic variability in terms of the disease course, lesion distribution, therapeutic response to IFNβ, and pathological aspects, suggesting that MS is a kind of neurological syndrome caused by different immunopathological mechanisms leading to the final common pathway that provokes inflammatory demyelination. DNA microarray technology is a novel approach to systematically monitoring the expression of a large number of genes. It gives us new insights into the complexity of molecular interactions promoting the autoimmune process in MS. By microarray analysis followed by hierarchical clustering analysis, we found that T cell gene expression profiling is valuable to identify distinct subgroups of MS associated with differential disease activity and therapeutic response to IFNβ. These observations suggest that microarray analysis is highly valuable for designing personalized treatment for heterogeneous populations of MS.

Key words : DNA microarray, gene expression profile, multiple sclerosis, personalized medicine

はじめに

多発性硬化症（multiple sclerosis; MS）は中枢神経系白質に炎症性脱髄巣が多発し，様々な神経症状が再発を繰り返して進行する難病である。MS 発症機序は十分解明されていないが，多数の遺伝的要因と環境因子の存在下で，脳炎惹起性髄鞘抗原に分子相同性を示すウイルスなどの外来抗原を認識し活性化した自己反応性 $CD4^+$ Th1 T 細胞が，血液脳関門を通過して中枢神経系組織内に浸潤し，マクロファージ・ミクログリアを活性化して TNFα などの炎症増強因子の産生を誘導し，脱髄が惹起されると考えられている（自己免疫機序）[1)]。回復期には髄鞘再生を認めるが，炎症が高度で遷延化すると髄鞘再生不全・軸索傷害・神経変性を来して不可逆的な機能障害を残す。近年欧米・本邦で実施された大規模臨床試験により，インターフェロンベータ（interferon-beta; IFNβ）の MS 再発抑制効果が立証され，現在では急性増悪期に副腎皮質ステロイド短期間大量静脈内投与を行い，回復期に IFNβ の継続的皮内・筋肉内投与を行う方法が，最も一般的な治療法として選択されている。しかし IFNβ が全く効果を示さない症例も多い[2,3)]。すなわち MS は均一な疾患ではなく多様な病態（phenotypic heterogeneity）を呈する疾患群である可能性が高い。実際 MS は臨床経過から再発寛解型（relapsing-remitting MS; RRMS），2 次進行型（secondary-progressive MS; SPMS），1 次進行型（primary-progressive MS; PPMS），病巣分布から脳型（conventional MS; CMS）と視神経脊髄型（opticospinal MS; OSMS），

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IFNβ 治療反応性から responder（RMS），nonresponder（NRMS）に分類される。病理学的にもT細胞浸潤，抗体沈着，oligodendrocyte apoptosis の観点から4型に分類される[4]。近年 MS の免疫病態の多様性を解析する手法として遺伝子アレイが用いられている。ヒトゲノムプロジェクトの完結によりヒト全遺伝子の塩基配列が解明された結果，遺伝子アレイを用いて個々の細胞における数万遺伝子（ヒト全遺伝子約30,000）の発現情報を包括的・網羅的・系統的に解析可能になった。RNA 発現解析を transcriptome 解析，タンパク質発現解析を proteome 解析と呼ぶ。網羅的発現解析（global expression analysis）により，従来の少数分子に焦点を向けた研究（pinpoint study）では予期しなかった遺伝子群の MS 発症機序における役割が次々明らかになった[5]。また治療による遺伝子発現変化を経時的に解析することにより薬物反応性遺伝子を同定し（薬理ゲノミクス pharmacogenomics），有効性や副作用を治療開始前に予知することにより，テーラーメイド医療（personalized medicine）に道が開かれた。本稿では DNA マイクロアレイ解析の基本原理と MS の免疫病態解析における応用に関して最近の知見を概説する。

1. DNA マイクロアレイ解析の基本原理

遺伝子アレイはスライドグラスやナイロン膜などの基盤上に，機能既知または未知の数千・万の cDNA または oligonucleotide が貼付けてあるチップである。主としてcDNAをスポッターで基盤上にスポットしてあるDNAマイクロアレイ（DNA microarray）と光オリゴヌクレオチド合成により基盤上で直接高密度の oligonucleotide を伸長合成している GeneChip（Affymetrix）に分類される（表1）[6]。スライドグラスを DNA microarray，ナイロン膜を DNA macroarray と総称することもある。最近では約3,000種類のタンパク質をスライドグラスに固定してあるプロテインチップ（protein microarray）も普及しており，タンパク質間相互作用（interactome）やシグナル伝達系の網羅的解析に用いられている[7]。遺伝子アレイは遺伝子多型・変異解析にも応用可能であるが本稿では割愛する。

遺伝子アレイ解析ではまず遺伝子発現レベルの異なる2種類以上の細胞・組織，例えば IFNβ 投与前後の細胞などから mRNA を抽出し増幅する（図1）。DNA マイクロアレイでは一般的に別々の蛍光色素（Cy3，Cy5）でラベルした cDNA または cRNA を作成して同一チップ上で競合的ハイブリダイゼーションを行い，2色法と呼ばれる。GeneChip では in vitro transcription（IVT）により cDNA から biotin 標識 cRNA を作成，fragment に切断してハイブリダイゼーションを行い，streptoavidin-phycoerythrin（SAPE）を添加して蛍光標識する。GeneChip では1サンプルに1枚のアレイが必要で，アレイ間の比較になる。どちらの場合もスキャナーで蛍光シグナルを検出し，得られたデータ（dataset）を正規化（normalization）して統計学的検定を行い，サンプル間の遺伝子発現プロフィール（gene expression profile）を比較解析する。同

表1 cDNA microarray と GeneChip の比較

	cDNA/Oligonucleotide Microarray	GeneChip
基盤	スライドグラス（microarray）またはナイロン膜（macroarray）	半導体チップ
固定化法	スポッティングまたは化学合成	オンチップフォトリソグラフ合成
遺伝子	300–1000 bp cDNA or 30–80 mer oligonucleotide	25 mer oligonucleotides of perfect match (PM) and mismatch (MM)
集積度	40,000/slide 程度	>500,000/chip
Tm	不均一	一定
蛍光標識	2色法（Cy3，Cy5）	単色法
定量原理	競合的ハイブリダイゼーションによる比較	個々のチップのデーターを正規化して比較
代表的なヒト遺伝子発現解析用アレイ（遺伝子数；Commercial Supplier）	Whole Human Genome G4112A Array (41,000; Agilent), Human Whole Genome Bioarray (55,000; Amersham)	Human Genome U133 Plus 2.0 Array (47,000; Affymetrix)
カスタム性	高い 汎用マイクロアレイスキャナーが使用可能	低い 専用のハイブリダイゼーションオーブンや洗浄装置とスキャナーが必要

文献6より引用改変．

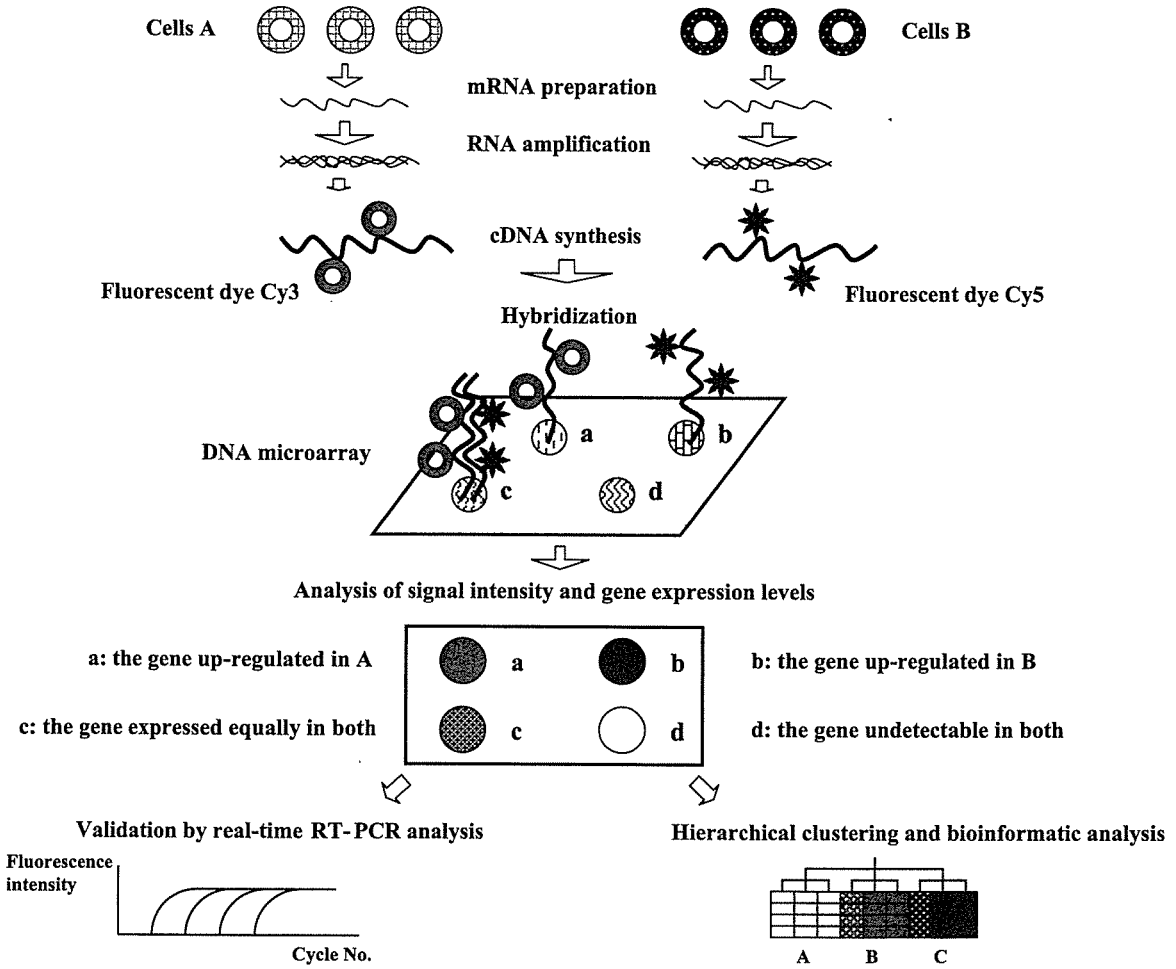

図1 DNAマイクロアレイ解析の概要。
2種類の細胞からmRNAを抽出増幅し，別々の蛍光色素（Cy3, Cy5）でラベルしたcDNA probeを作成して，cDNAがスポットされたチップ上で競合的ハイブリダイゼーションを行う。スキャナーで蛍光シグナルを検出し，データを正規化して統計学的検定を行い，サンプル間の遺伝子発現プロフィール（gene expression profile）を比較解析する。さらに階層的クラスター解析（hierarchical clustering analysis）を行って，類似した発現パターンを呈する遺伝子やサンプルをグループに分類する。有意な発現差異を呈する遺伝子はreal-time RT-PCRでmRNAを定量して検証する。

定した遺伝子の機能・構造の注釈情報（annotation）は遺伝子リストのIDからWeb上でdatabaseを検索可能である（表2）。既に様々な細胞・組織の遺伝子発現データがGene Expression Omnibus（GEO; www.ncbi.nlm.nih.gov/geo）に登録されているが，実験に用いたチップのformatが異なるとデータ間の互換性がなくなると考えられ，大規模なmeta-analysisを実施する場合に支障となる[8]。サンプル数が多い場合はデータセットの要素の特性を抽出するため，階層的クラスター解析（hierarchical clustering analysis）を行う[9]。すなわちサンプルに関する事前情報なしに（教師なし法 unsupervised method），類似した発現パターンを呈する遺伝子やサンプルをグループに分類して，樹状図（dendrogram）と発現レベルの2次元マトリックスで表示する。またサンプルをいくつかのグループに分類する代表な遺伝子（discriminator genes）を抽出し，これらを3次元に圧縮投射する主成分解析（principal component analysis）を行う[9]。

我々は薬物応答遺伝子アレイ 1,258 cDNA microarray（Hitachi Life Science）を用いて，MS患者末梢血T細胞の遺伝子発現プロフィールを解析している。健常者3名のRNA mixtureをuniversal referenceとしてCy3で標識し，患者や健常者のサンプルを全てCy5で標識し，各サ

表2 Transcriptome・proteome 解析に有用な database

Name	Website	Contents
★統合 Database 検索システム		
Entrez	www.ncbi.nlm.nih.gov/Entrez/index.html	The Life Sciences Search Engine
Gene	www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=gene	A Searchable Database of Genes
OMIM	www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=omim	Online Mendelian Inheritance in Man
DBGET	www.genome.ad.jp/dbget/dbget.links.html	Web of Molecular Biology Databases
KEGG	www.genome.ad.jp/kegg	Kyoto Encyclopedia of Genes and Genomes
SRS	srs6.ebi.ac.uk	European Bioinformatics Institute Database
HGMD	www.hgmd.cf.ac.uk/hgmd0.html	Human Gene Mutation Database
★配列解析		
UniProt	www.genome.jp/dbget-bin/www_bfind?uniprot	SWISS-PROT Protein Sequence Database
PIR	www.genome.jp/dbget-bin/www_bfind?pir	PIR Protein Sequence Database
BLAST	blast.genome.jp	Sequence Similarity Search
dbSNP	www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=snp	Single Nucleotide Polymporphism Database
CLUSTALW	align.genome.jp	Multiple Sequence Alignment
TraceSuite II	www-cryst.bioc.cam.ac.uk/~jiye/evoltrace/evoltrace.html	Evolutionary Trace Server
ORF Finder	www.ncbi.nlm.nih.gov/gorf/gorf.html	Open Reading Frame Finder
PROSCAN	thr.cit.nih.gov/molbio/proscan	Web Promoter Scan
★タンパク質立体構造解析		
PDB	www.rcsb.org/pdb	The RCSB Protein Data Bank
RasMol	www.rcsb.org/pdb/help-graphics.html#rasmol_download	Molecular Graphics
GRASS	honiglab.cpmc.columbia.edu/cgi-bin/GRASS/surfserv_enter.cgi	Graphical Representation and Analysis of Structure Server
SWISS-MODEL	swissmodel.expasy.org//SWISS-MODEL.html	An Automated Comparative Protein Modelling Server
ERRAT	nihserver.mbi.ucla.edu/ERRAT	A Protein Structure Verification Algorithm
Verify3D	nihserver.mbi.ucla.edu/Verify_3D	A Crystal Structure Evaluation Server
SCOP	scop.mrc-lmb.cam.ac.uk/scop/index.html	Structural Classification of Proteins
DBAli	salilab.org/DBAli	A Database of Structure Alignments
★タンパク質機能予測		
SOSUI	sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html	Classification and Secondary Structure Prediction of Membrane Proteins
PSORT II	psort.ims.u-tokyo.ac.jp	Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences
SignalP 3.0	www.cbs.dtu.dk/services/SignalP	Prediction of Signal Peptide Cleavage Sites in Amino Acid Sequences
InterPro	www.ebi.ac.uk/interpro	A Database of Protein Families, Domains and Functional Sites
PredictProtein	www.embl-heidelberg.de/predictprotein/predictprotein.html	Structure Prediction and Sequence Analysis
BIND	www.bind.ca	The Biomolecular Interaction Network
DIP	dip.doe-mbi.ucla.edu	Databases of Interacting Proteins
MINT	160.80.34.4/mint/index.php	A Molecular Interaction Database
PPID	www.anc.ed.ac.uk/mscs/PPID/cgi-bin/ppid_search.pl	Protein-Protein Interaction Database
PROCAT	www.biochem.ucl.ac.uk/bsm/PROCAT/PROCAT.html	A Database of 3D Enzyme Active Site Templates
Scansite	scansite.mit.edu	A Motif Scan
PhosphoSite	www.phosphosite.org/Login.jsp	An In Vivo Phosphorylation Site Database
ExPASy	au.expasy.org	Expert Protein Analysis System Proteomics Server

2005年8月の時点でのサイト．

ンプルで遺伝子ごとに Cy5/Cy3 signal intensity ratio を測定している。比較する2群間（MS vs 健常者など）で有意な発現差異を呈する遺伝子は，Bayesian t test または R 解析（www.cran.r-project.org）と Bonferroni 補正で統計学的有意性を検定することにより同定している。さらに有意な遺伝子に関しては，LightCycler（Roche）による

real-time RT-PCRで定量してアレイ解析の結果を検証（validation）している。階層クラスター解析と主成分解析は GeneSpring（Silicon Genetics-Agilent）で行っている。末梢血リンパ球の遺伝子アレイ解析の問題点は，特定の遺伝子の発現レベルが年齢・性・喫煙・飲酒・常用薬・嗜好品・精神的ストレスなどの個人差や採血時刻（日内変動）の影響を受けることである（interindividual and intraindividual variation）[10]。また脳組織の遺伝子アレイ解析の問題点は死後脳凍結までに要する時間（RNA degradation time）で，組織の pH がある程度参考になる。

2. DNA マイクロアレイによる多発性硬化症の免疫病態の解析

2.1. MS 脳組織の網羅的遺伝子発現解析

DNA マイクロアレイによる MS の病態解析の最初の報告は Whitney らによる研究である[11]。彼らは独自の cDNA microarray を用いて MS 急性期炎症性病巣と正常白質（normal-appearing white matter; NAWM）を比較し，MS 病巣における interferon-regulatory factor IRF-2, 5-lipooxygenase 発現上昇を報告した（表 3）[11,12]。Chabas らは MS brain cDNA ライブラリーの網羅的シークエンス解析で osteopontin（OPN）発現レベルの上昇を認めた[13]。さらにラット EAE 脊髄のカスタム oligonucleotide microarray 解析で OPN の発現上昇を確認した。OPN は主として T 細胞が産生し macrophages による IL-12 産生を促進して IL-10 産生を抑制する Th1 cytokine で，活動性 RRMS 患者血清で上昇している[14]。OPN 遺伝子欠損マウスは EAE 惹起に対して抵抗性を示す[13]。Lock らは GeneChip を用いて MS 急性炎症性病巣と慢性非活動性病巣を比較し，活動性病巣での granulocyte colony stimulating factor（G-CSF）発現上昇と非活動性病巣での IgG Fc receptor, IgE receptor, histamine receptor type 1 の発現上昇を認めた[15]。さらに彼らは G-CSF 投与で EAE を軽症化出来ること，immunoglobulin FcR γ-chain 遺伝子欠損マウスでは EAE 慢性化が抑制されることを証明し，アレイ解析の結果を裏付けた。Chabas らや Lock らの報告により，MS, EAE の病巣形成における allergic response mediators の役割が認識されるようになった[16]。

Mycko らは cDNA microarray（Clontech）を用いて SPMS の慢性活動性病巣と非活動性病巣，脱髄巣辺縁部と中心部を比較し，活動性病巣辺縁部における炎症・免疫応答遺伝子群（TNFα など）の発現上昇を認めた[17]。Graumann らは cDNA macroarray（Clontech）を用いて MS の NAWM と非神経疾患のコントロール白質を比較し，NAWM における脳虚血関連遺伝子（hypoxia-inducible factor 1 alpha; HIF-1α など）の発現上昇を認めた[18]。Lindberg らは GeneChip で SPMS の活動性病巣と NAWM を比較し，活動性病巣での immunoglobulin 産生亢進の所見を見出した[19]。Tajouri らは独自の cDNA microarray を用いて SPMS の急性・慢性活動性病巣を non-MS コントロール白質と比較し，活動性病巣における αB-crystallin, superoxide dismutase SOD1 の発現上昇を報告した[20]。上述の MS 脳組織のマイクロアレイ解析は各々症例数が少なく，RNA 抽出部位が必ずしも全体像を反映していない可能性は否定出来ない。EAE 脳・脊髄の網羅的遺伝子発現解析に関しては割愛する[21-23]。

2.2. 末梢血リンパ球を用いた MS と健常コントロールの比較解析

Ramanathan らは Research Genetics（Invitrogen）の GeneFilter membrane array を用いて，MS と健常者の monocyte-depleted peripheral blood lymphocytes（PBL）を比較し，MS における lymphocyte-specific protein tyrosine kinase（LCK），IL-7R の発現上昇を報告した[24]。LCK は Airla らによる RRMS の PBMC の cDNA macroarray（Clontech）解析で，intravenous methylprednisolone pulse（IVMP）で発現低下する遺伝子として報告されている[25]。Bomprezzi らは独自の cDNA microarray を用いて，24 例の RRMS と 21 名の健常者の peripheral blood mononuclear cells（PBMC）を比較し，発現差異を示した 53 遺伝子を同定した[26]。MS では自己反応性 T 細胞活性化に関連する IL-7R, ZAP70, TNFRSF7（CD27）の発現上昇およびサイトカイン mRNA の ubiquitin-proteasome 系による分解を制御する HSPA1A（HSP70）の発現低下を認めた。Mayne らは RRMS と健常者の CD4陽性T細胞を negative selection で分離し，cDNA membrane array（NIA）を用いて解析し，MS における cytoplasmic FMR1 interacting protein 2（CYFIP2）の発現上昇を認めた[27]。

我々は cDNA microarray（Hitachi Life Science）を用いて，72 例の MS（65 RRMS, 7 SPMS）と 22 名の健常者の末梢血 CD3 陽性 T 細胞，CD3 陰性 non-T 細胞の遺伝子発現プロフィールを解析した（表 3）[28]。その結果 T 細胞で 173 遺伝子，non-T 細胞で 50 遺伝子の発現差異を認め，上位 30 遺伝子（the most significant genes）を抽出すると，T 細胞で 25 遺伝子（NR4A2, TCF8 の上昇と

表3 Microarrayによる MS の免疫病態の解析

Authors (Reference No.)	Year	No of MS Patients and Controls	RNA Samples
Whitney et al. (11)	1999	PPMS (n=1)	acute lesion vs NAWM
Ramanathan et al. (24)	2001	RRMS (n=15) vs HC (n=15)	monocyte-depleted PBL
Wandinger et al. (35)	2001	RRMS (n=1) plus HC (n=2)	PBMC incubated with IFNβ in vitro
Whitney et al. (12)	2001	PPMS (n=1), RRMS (n=1), EAE vs HC (n=3)	acute or chronic lesions of MS and EAE vs white matter of non-MS controls
Lock et al. (15)	2002	CPMS and SPMS (n=4)	acute or chronic active lesions vs chronic silent lesions
Mass et al. (32)	2002	RA (n=20), SLE (n=24), IDDM (n=5), and MS (n=5) vs HC before and after influenza vaccination (n=9)	PBMC
Bomprezzi et al. (26)	2003	RRMS (n=18), SPMS (n=6) vs HC (n=21)	PBMC (fresh or frozen)
Graumann et al. (18)	2003	SP/PP/RRMS (n=10) vs non-neurological controls (n=7)	NAWM vs control white matter
Koike et al. (36)	2003	RRMS (n=13) before and at 3 and 6 months after IFNβ treatment	T and non-T cells separated from PBMC
Mycko et al. (17)	2003	SPMS (n=4)	chronic active vs silent lesions and the lesion margin vs center
Stürzebecher et al. (46)	2003	RRMS before and after IFNβ treatment for 6 months (n=10; 6 responders vs 4 non-responders)	frozen PBMC ex vivo or incubated with IFNβ in vitro
Tajouri et al. (20)	2003	SPMS (n=5) vs non-MS	acute and chronic active lesions
Weinstock-Guttman et al. (44)	2003	RRMS before and at 1, 2, 4, 8, 24, 120, and 160 h after IFNβ treatment (n=8)	monocyte-depleted PBL
Achiron et al. (29)	2004	RRMS (n=26; 14 with treatment) vs HC (n=18)	PBMC
Achiron et al. (30)	2004	RRMS treated (n=13) vs untreated (n=13)	PBMC
Airla et al. (25)	2004	RRMS (n=6) before and after IVMP	PBMC
Hong et al. (47)	2004	RRMS/SPMS treated with IFNβ (n=18), GA (n=12) or untreated (n=15)	PBMC
Iglesias et al. (33)	2004	RRMS (n=17) vs HC (n=7)	PBMC
Lindberg et al. (19)	2004	SPMS (n=6) vs non-neurological controls (n=12)	active lesions vs NAWM
Mandel et al. (31)	2004	RRMS (n=13) vs SLE (n=5) vs HC (n=18)	PBMC
Mayne et al. (27)	2004	RRMS (n=21) vs HC (n=19)	$CD4^+$ T cells
Satoh et al. (28)	2005	RRMS (n=65) plus SPMS (n=7) vs HC (n=22)	T and non-T cells separated from PBMC

Abbreviations: RRMS, relapsing-remitting MS; SPMS, secondary progressive MS; PPMS, primary progressive MS; CPMS, chronic progressive MS; HC, healthy controls; IDDM, insulin-dependent diabetes mellitus; NAWM, normal appearing white matter; PBMC, peripheral blood mononuclear cells; PBL, peripheral blood lymphocytes; IFN, interferon; GA, glatiramer acetate; IVMP, intravenous methylprednisolone pulse.

MAPK1, SMARCA3, HSPA1A, TRAIL, TOP1, CCR5, BAG1, DAXX, TSC22, PARP の低下など), non-T 細胞で27遺伝子 (ICAM1, CDC42, RIPK2, SODD, TOP2A の上昇と BCL2, RPA1, NFATC3, HSPA1L, RBBP4, PRKDC の低下など) が apoptosis 制御遺伝子に属していた。すなわち apoptosis 促進遺伝子 (proapoptotic genes) と抑制遺伝子 (antiapoptotic genes) の発現上昇・低下の拮抗的バランス (counterbalance) を認め, MS 免疫病態における apoptosis 制御機構の異常が示唆された。Achiron らは GeneChip を用いて, 26 例の RRMS と 18 名の健常者の PBMC を比較解析した[29]。両群間で 1,109 遺伝子の発現差異を認め, MS における T 細胞活性化関連遺伝子

Type of Microarray	No of Genes on Microarray	Key Findings
Original cDNA Glass Array	1,344 or 5,000	Upregulation of IRF-2 and TNFRp75 in acute lesions
GeneFilters GF211 Membrane Array (Research Genetics)	5,184	Upregultion of LCK, IL-7R and MMP-19 and downregulation of CCR6 and DFFA in MS
Mini-Lymphochip cDNA Array	6,432	Upregulation of proinflammatory genes such as CCR5, IP-10, and IL-15RA by IFNβ treatment
Original cDNA Glass Array	2,798	Upregulation of 5-lipoxygenase in MS and EAE lesions
HuGene FL Oligonucleotide Array (Affymetrix)	7,026	Upregulation of G-CSF in active lesions and upregulation of IgG FcR in silent lesions, and amelioration of EAE in FcRγ-chain-KO mice and by treatment with G-CSF
GeneFilters GF211 Membrane Array (Research Genetics)	4,329	Indistinguishable profiles between MS and IDDM and downregulation of apoptosis-regulatory genes in autoimmune diseases
Original cDNA Array (Modified Lymphochip)	6,500 or 7,500	Upregulation of PAFAH1B1, IL-7R, ZAP70, and TNFRSF7(CD27) and downregulation of HSPA1A (HSP70) and CKS2 in MS
Atlas Human cDNA Memebrane Array 1.2 (Clontech)	3,528	Upregulation of ischemic preconditioning genes such as HIF-1α in NAWM of MS
Human cDNA Array (Hitachi Life Science)	1,258	Upregulation of 15 IFN-responsive genes in MS after IFNβ treatment
Atlas Human 1.0 Glass Microarray (Clontech)	588	Upregulation of inflammation/immune-related genes in the margin of active lesions
Mini-Lymphochip cDNA Array	6,432 or 12,672	Downregulation of IL-8 in responders after IFNβ treatment
Custom-made cDNA Glass Array	5,000	Upregulation of αB-crystallin and SOD in acute lesions
GeneFilters GF211 Membrane Array (Research Genetics)	5,184	Time-dependent upregulation of IFN-responsive genes
Human U95Av2 Oligonucleotide Array (Affymetrix)	12,000	Upregulation of T cell activation genes and downregulation of IL-1 and TNF signaling genes in MS
Human U95Av2 Oligonucleotide Array (Affymetrix)	12,000	Identification of SCYA4, IL2RG, and TNFRSF6(Fas) as immunomodulatory treatment-associated genes
Atlas Human Hematology/Immunology Membrane Array (Clontech)	448	Downregulation of LCK, TCF7, CD5, and ISGF3 by IVMP
Original Membrane Array	36	Distinct gene expression profile between MS patients treated with IFNβ and GA
HuGene FL Oligonucleotide Array (Affymetrix)	6,800	Upregulation of E2F transcription factor pathway genes in MS
Human U95A Oligonucleotide Array (Affymetrix)	12,633	Upregulation of genes related to Ig synthesis in active lesions of MS
Human U95Av2 Oligonucleotide Array (Affymetrix)	12,000	Downregulation of NR4A1 and NR4A3 as the autoimmunity-specific signature
Immune Membrane Array (National Institute on Aging)	1,152	Upregulation of CYFIP2 in MS
Human cDNA Array (Hitachi Life Science)	1,258	Aberrant expression of apoptosis and DNA damage-regulatory genes in MS

(LEF1, TCF3, SLAM, ITGB2, CTSB) の発現上昇および IL-1β, TNFα シグナル伝達系遺伝子の発現低下を認めた。我々の結果[28]に反し, MS における orphan nuclear receptor NR4A2 の発現低下を報告した。彼らの研究では MS 14 例は採血時に IFNβ, glatiramer acetate (GA), IVIg 治療中である点が問題である。彼らは同じ症例の治療中13例と未治療13例のPBMCの比較解析を行い, 治療関連7遺伝子 (TNFRSF6; Fas など) を同定した[30]。さらに13例の RRMS と 5 例の SLE を 18 名の健常者と比較して, 自己免疫疾患共通遺伝子 (autoimmunity-specific signature) を探索し, 自己免疫疾患における apoptosis, matrix metalloproteinase (MMP) 制御系遺伝子の発現異

常を発見した[31]。Maasらも20例のRA, 24例のSLE, 5例のIDDM, 5例のMSと9名の健常者（influenzaワクチン接種前後）のPBMCを比較解析した[32]。ワクチンに対する免疫応答と自己免疫疾患の遺伝子発現プロフィールは全く異なるが，RAとSLE，MSとIDDMは極めて類似し，自己免疫疾患では共通してapoptosis制御遺伝子群の発現低下を認めることを報告した。Iglesiasらは GeneChipを用いて17例のRRMSと10名の健常者のPBMCを比較し，MSにおけるE2F transcription factor pathway遺伝子群の発現上昇を見出し，E2F1遺伝子欠損マウスではEAEが軽症化することを報告した[33]。

2.3. MSにおけるインターフェロンベータ治療反応性の解析

我々はcDNA macroarray（Invitrogen）を用いて，ヒト胎児脳より樹立したアストロサイト（astrocytes）純培養でIFNβ，IFNγにより発現変動する遺伝子群を解析した[34]。IFNβによるinterferon-regulatory factor IRF-7とpleiotrophinの発現上昇，IFNγによるIRF-1とICAM-1の発現上昇を発見した。Wandingerらは RRMSと健常者のPBMCをIFNβで刺激してcDNA microarray（Mini-Lymphochip）を用いて解析した[35]。彼らはproinflammatory moleculesであるCC chemokine receptor 5（CCR5），interferon-inducible cytokine IP-10（CXCL10），IL15 receptor alpha（IL-15RA）の発現上昇を認めた。我々はcDNA microarray（Hitachi Life Science）を用いて，13例のRRMSの末梢血CD3陽性T細胞とCD3陰性non-T細胞で，IFNβ1b治療開始後に発現変動したインターフェロン応答遺伝子群（IFN-responsive genes; IRG）を同定した[36]。21遺伝子が有意な変動を呈し，T細胞で8 IRG（IRF-7, ISG15, IFI56, IFI6-16, IFI60, IFI30, ATF3, TLR5）の発現上昇，IL-3，monokine induced by IFNγ（MIG）などの発現低下を認め，non-T細胞では12 IRG（IRF-7, ISG15, IFI56, IFI6-16, IFI27, IFI17, TAP1, TNFAIP6, TSC22, SULT1C1, RPC39, RAB11A）の発現上昇，IL-3の発現低下を認めた。ISG15, IFI56, IFI6-16, IFI27, TSC22, SULT1C1に関しては，治療開始後3–6ヶ月の持続的な上昇を認めた。一方統計学的有意差は見られなかったが，治療後にTh1関連遺伝子CCR5（T），IFNγ（T），TNFα（non-T）の発現上昇傾向を認めた。このことはMSにおいてIFNβ治療は必ずしも明確なTh2 shiftを誘導しないという見解[35]に一致する。上記のうち9遺伝子（IRF7, ISG15, IFI56, IFI6-16, IFI60, IFI17, TAP1, TNFAIP6, MIG）はプロモーター領域にIFN-stimulated response element（ISRE）やIRF element（IRF-E）が同定されている既知のIRGであり，IFNβ治療に直接反応して上昇し治療効果発現に深く関与していると考えられる。興味深いことに培養系では多くのIRGはIFNγによっても発現が誘導される[36,37]。IRF-7はウイルス感染時にIFNα/β産生を増幅する正の制御因子である[38]。IFI30はclass II MHC拘束性抗原提示に働くチオール還元酵素であり，IFI30遺伝子欠損マウスでは抗原呈示能低下を来す[39]。TAP1はclass I MHC拘束性抗原提示を司るペプチド輸送因子で，TAP1遺伝子欠損マウスでは$CD8^+$ T細胞を介する結核菌への抵抗力が減弱する[40]。TNFAIP6はTNFα, IL-1βにより発現誘導される分泌蛋白質で，マウス関節炎に投与すると抗炎症作用を呈する[41]。以上よりMSにおいてIFNβはantiviral and antiinflammatory mediator遺伝子群の発現上昇を誘導することが明らかになった。非常に興味深いことにSLEでは治療に関わらずPBMCにおけるIRGの発現レベルが高い[42,43]。

Weinstock-Guttmanらは GeneFilter membrane arrayを用いて，8例のRRMSでIFNβ1a投与後経時的にmonocyte-depleted PBLを解析してIRGを同定したが，その多くは我々の結果とオーバーラップしている[44]。またLiangらはWeinstock-Guttmanらのデータを再解析し，IFNβにより発現誘導されるIRGは early-onset（within 8 hours），intermediate-onset（24 hours），late-onset（48 hours）の3群に分類されることを報告した[45]。Stürzebecherらは cDNA microarray（Mini-Lymphochip）を用いて，10例のRRMSでIFNβ1b治療前後のPBMCを解析した[46]。治療開始前6ヶ月から開始12ヶ月後まで毎月Gd造影MRIで活動性病巣数を算出し，治療後に病巣数が60%以上減少した症例をresponderと定義した。またnonresponderを治療開始後から効果のない nonresponder from initiation of therapy（INR）と，治療開始後一定期間は効果がありneutralizing antibody（NAb）出現とともに効果が減弱したnonresponder with development of NAb（NAbNR）の2群に分類した。さらにex vivo解析と同時にIFNβで刺激したin vitro解析も行った。Responderで治療後2倍以上変動した遺伝子はex vivoでは25遺伝子（IFI17, OAS, Stat1の上昇とIL-8, CD69, c-fos, TSC22の低下など）で，そのうちIL-8発現低下はresponderの指標となる可能性が示唆された。一方in vitro IRGは87遺伝子で，responderと nonresponderの間で発現レベルに差異を認めなかっ

た。彼らの結果に反して，我々は IFNβ 治療後に non-T 細胞で TGFβ-stimulated protein TSC22 の発現上昇を認めている[36]。彼らの研究の問題点は responder 6 例・INR 2 例・NAbNR 2 例と症例数が少なく，PBMC を凍結保存後に解凍して刺激しており，実験操作で遺伝子発現が変化し得ることである[46]。さらに 1 例の responder では治療前に約 90 個の Gd 造影病巣を呈しているが，これほど多数の造影病巣を示す症例は日本人 MS では極めて異例である。Hong らは免疫応答に極めて重要な 36 遺伝子に絞った cDNA macroarray を作成し，未治療 MS と IFNβ1a または GA 治療症例の PBMC を解析し，治療反応性遺伝子群の相違を明らかにした[47]。興味深いことに活性化 T 細胞の血液脳関門通過に重要な MMP-9 の発現は IFNβ により低下したが GA では上昇した。

van Boxel-Dezire らは 26 例の IFNβ1b 治療中の RRMS で PBMC のサイトカイン遺伝子発現レベルを半定量的 RT-PCR で経時的に解析した[48]。治療前後 2 年間の再発回数・IVMP 回数・Extended Disability Status Scale (EDSS) スコアから 16 例の responder と 10 例の nonresponder に分けて比較すると，responder は治療前に IL-12p35 発現レベルが低い傾向を呈した。Wandinger らは RRMS で IFNβ1a 治療後 1 年間一度も再発がなく，EDSS スコア悪化の見られない症例を responder, 一度以上再発が見られた症例を nonresponder と定義し，20 例の responder と 19 例の nonresponder の PBMC を比較し，responder では TNF-related apoptosis-inducing ligand (TRAIL; TNFSF10) が持続的高値を呈することを報告した[49]。TRAIL は IRG の 1 つで我々は MS の T 細胞における発現低下を認めている[28]。TRAIL 遺伝子欠損マウスは胸腺細胞の apoptosis に異常を来たし，コラーゲン関節炎などに高感受性になることが報告されている[50]。Baranzini らは 52 例の IFNβ 治療中の RRMS で経時的に PBMC の 70 遺伝子の発現レベルを RT-PCR で定量的に解析した。彼らは治療後 2 年間一度も再発がなく，EDSS スコア悪化の見られない症例を responder, 2 回以上再発が見られた症例を nonresponder と定義し，両者は 3 遺伝子 (caspase2, caspase10, FLICE inhibitory protein; FLIP) の発現レベルの 3 次元解析で 86%区別可能と報告している[51]。

最近我々は前述[28]の 72 例の IFNβ 未治療 MS (46 例は初回採血後 2 年間 IFNβ 治療開始) と 22 名の健常者の末梢血 CD3 陽性 T 細胞を cDNA microarray (Hitachi Life Science) を用いて解析したデータを，両群間で発現差異を示す 286 遺伝子を指標にして階層的クラスター解析で再解析した (Satoh et al. Manuscript in preparation)。この解析により 286 遺伝子は class #1–#5 に分類され，MS は健常者から分離されてさらに 4 つのサブグループに分類された。すなわち遺伝子発現プロフィールが健常者に近似した A 群，治療導入前後 2 年間の再発回数・IVMP 日数・入院日数の点で最も活動性が高い B 群，大脳限局病変が多い C 群，最も EDSS スコアが高値の D 群に分類された。B 群は chemokine 遺伝子を多く含む class #5 の発現レベルが高かった。また IFNβ 治療前後 2 年間の再発回数・IVMP 日数・入院日数・EDSS スコア・MRI T2 強調画像病巣数の比較と患者満足度から算出した IFNβ 治療反応スコアで評価すると，responder は A 群と B 群に集積していた。また responder では nonresponder に比較して治療開始後 6ヶ月の時点でも IRG (ISG15, IFI27, MCP-1, TNFRp75) の発現レベルが高く保持される傾向を示した。

3. 結　語

我々は DNA マイクロアレイ解析を用いて MS が T 細胞の遺伝子発現プロフィールに基づき 4 群に分類され，各群は疾患活動性・病変分布・IFNβ 治療反応性との対応を認めることを報告した。現在，欧米人 MS にも同様の結果が当てはまるかどうか症例数を増加して解析中である。このような研究成果を積み重ねることにより MS のテーラメイド医療樹立に貢献出来ると思われる。

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