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要　　旨

多発性硬化症（multiple sclerosis; MS）発症は多数の遺伝因子と環境因子の複雑な相互作用により規定されている。そのため MS は臨床経過・病巣分布・治療反応性・病理学的所見の点から多様な病態（clinical heterogeneity）を呈する。遺伝子アレイ（DNA microarray, GeneChip）は基盤上に数万遺伝子が貼付けてあるチップである。遺伝子アレイによる MS 患者末梢血リンパ球や脳組織の網羅的遺伝子発現解析は，MS の分子遺伝学的発症機序解明に威力を発揮する。特に遺伝子アレイ解析により従来の研究方法では予期しなかった遺伝子群の MS 病態における重要な役割を発見したり，インターフェロン応答遺伝子群（IFN-responsive gene; IRG）を同定することにより治療有効性や副作用を事前に予知することが可能になりつつある。最近我々は階層的クラスター解析（hierarchical clustering analysis）により，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・インターフェロン（IFNβ）治療反応性と密接な対応を認めることを発見した。遺伝子アレイ解析は MS のテーラーメイド医療（personalized medicine）樹立に道を開くと思われる。

キーワード：遺伝子発現プロフィール，DNA マイクロアレイ，多発性硬化症，テーラーメイド医療

網羅的遺伝子発現解析による多発性硬化症の
病態・薬物反応性

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MSの発症は多数の遺伝因子と環境因子の複雑な相互作用により規定されており，臨床経過・病巣分布・治療反応性・病理学的所見の観点から多様な病態(clinical heterogeneity)を呈する．遺伝子アレイ(DNA microarray/GeneChip)は基盤上に数万遺伝子が貼りつけてあるチップである．遺伝子アレイによるMS患者末梢血リンパ球・脳組織の網羅的遺伝子発現解析は，MS分子遺伝学的発症機序の解明に威力を発揮する．従来の研究方法では予期しなかった遺伝子群のMS病態における重要な役割を発見したり，インターフェロン応答遺伝子群(IFN-responsive genes：IRG)を同定して治療反応性や副作用を事前に予知することが可能になりつつある．最近われわれは階層的クラスター解析(hierarchical clustering analysis)により，MSがT細胞の遺伝子発現プロフィールにもとづき4群に分類され，各群は疾患活動性・病変分布・IFN-β治療反応性と密接な対応を認めることを見出した．遺伝子アレイ解析はMSのテーラーメイド医療の樹立に役立つと思われる．

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，さまざまな神経症状が再発をく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，遺伝的要因と環境因子の相互作用を背景に，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$^+$　Th1T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腫瘍壊死因子-α(tumor necrosis factor-α：TNF-α)などの炎症増強因子の産生を誘導し脱髄(demyelination)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，炎症が遷延化すると髄鞘再生不全・軸索傷害・神経変性をきたして不可逆的機能障害を残す．近年欧米およびわが国における大規模臨床試験により，インターフェロン(IFN)-β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(intravenous methylpredonisolone pulse：IVMP)をおこない，回復期にIFN-βの継続的皮内・筋肉内投与をおこなう方法が，最も一般的な治療法として選択されている．しかしIFN-βがまったく効果を示さない症例も多い[2]．すなわちMSは均一な疾患ではなく多様な病態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

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型(relapsing-remitting MS：RRMS)，二次進行型(secondary-progressive MS：SPMS)，一次進行型(primary-progressive MS：PPMS)，病巣分布から脳型(conventional MS：CMS)，視神経脊髄型(opticospinal MS：OSMS)，IFN-β 治療反応性からレスポンダー(応答)(responder MS：RMS)，ノンレスポンダー(非応答)(nonresponder MS：NRMS)に分類される．病理学的には T 細胞浸潤，抗体沈着，オリゴデンドログリアのアポトーシス(apoptosis)の所見により 4 型に分類される[3]．

近年 MS の免疫病態の多様性(heterogeneity)を解析する手段として遺伝子アレイ(DNA microarray/GeneChip)が用いられている．ヒトゲノムプロジェクトによりヒト全遺伝子塩基配列が解明され，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・系統的に解析することが可能になった．RNA 発現解析をトランスクリプトーム解析，蛋白質発現解析をプロテオーム解析とよぶ．このような網羅的発現解析(global expression analysis)により，従来の研究方法では予期し得なかった遺伝子群の MS 病態における役割がつぎつぎ明らかにされた[4]．また治療に反応する遺伝子群の変動を経時的に解析することにより，薬物反応性や副作用を予知することが可能になりつつある(薬理ゲノミクス，pharmacogenomics)．本稿では DNA マイクロアレイによる MS の免疫病態・薬物反応性の解析に関する最近の知見を概説する．

1．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，数千～一万の cDNA またはオリゴヌクレオチドが貼りつけてあるチップである．cDNA をスポッターで基盤上にスポットしてある DNA マイクロアレイと，基盤上で直接高密度オリゴヌクレオチドをフォトリソグラフ合成して

いる GeneChip(Affymetrix)に分類される．スライドグラスをマイクロアレイ，ナイロン膜をマクロアレイと総称する．まず遺伝子発現レベルが異なる 2 種類以上の細胞・組織，たとえば IFN-β 投与前後の細胞などから mRNA を抽出し増幅する．DNA マイクロアレイでは別々の蛍光色素(Cy 3，Cy 5)で標識した 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おこなう(2 色法)．GeneChip では *in vitro* transcription により cDNA から biotin 標識 cRNA を作成，断片的に切断してハイブリダイゼーションをおこない，streptoavidin-phycoerythrin(SAPE)を添加して蛍光標識する(1 色法)．GeneChip 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，得られたデータを正規化(normalization)し，統計学的解析(R 解析：www.cran.r-project.org など)をおこない，サンプル間の遺伝子発現プロフィール(gene expression profile)を比較する．したがって RNA の質(quality)が結果に非常に影響する．有意な発現差異を示す遺伝子はリアルタイム RT-PCR で定量・検証することが重要である(validation)．同定した遺伝子の機能・構造に関する情報(annotation)は，Web 上でデーターベース(NCBI Entrez：www.ncbi.nlm.nih.gov/Entrez/index.htm など)を検索する．すでにさまざまな遺伝子発現データが Gene Expression Omnibus(GEO：www.ncbi.nlm.nih.gov/geo)に登録されておりダウンロードできる．末梢血リンパ球(peripheral blood mononuclear cells：PBMC)の遺伝子アレイ解析の問題点は，遺伝子発現レベルが年齢・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)[5]．また脳組織の解析では死後脳凍結までに要する時間(RNA degradation time)が問題であり，組織 pH が参考になる．

複数サンプルの場合はデータセットの要素特性を抽出し分類するため, 解析ソフト (GeneSpring: Silicon Genetics-Agilent など)を用いて, 階層的クラスター解析 (hierarchical clustering analysis)をおこなう. すなわちサンプルに関する事前情報なしに, 類似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, 樹状図 (dendrogram)と発現レベルの二次元マトリックスで表示する (教師なし法: unsupervised method). またグループを特徴づける指標遺伝子 (discriminator genes)を抽出して三次元に投射する主成分解析 (principal component analysis: PCA)をおこなう. さらに指標遺伝子抽出に用いたデータを training set として機械学習し, 新規データセットにおけるサブグループを高次元空間上で線形判別可能な超平面 (hyperplane)を同定するサポートベクターマシン (support vector machine: SVM)解析をおこなう (教師あり法: supervised method).

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 (表1)

1) MS 脳組織の網羅的遺伝子発現解析

Whitney ら[6)7)]は独自の cDNA マイクロアレイを用いて MS 急性期炎症性病巣と正常様白質 (normal-appearing white matter: NAWM)を比較し, MS 病巣におけるインターフェロン制御転写因子 (interferon-regulatory factor: IRF)-2, 5-lipooxygenase 発現上昇を報告した. Chabas ら[8)]は MS 脳 cDNA ライブラリーの網羅的シークエンス解析により osteopontin (OPN)発現上昇を認め, ラット実験的自己免疫性脳脊髄炎 (experimental autoimmune encephalomyelitis: EAE)モデルの脊髄を用いたカスタムオリゴヌクレオチドマイクロアレイ解析で OPN 上昇を確認した. OPN 遺伝子欠損マウスは EAE 惹起に対して抵抗性を示す[8)]. Lock ら[9)]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,

前者の G-CSF 上昇と後者の IgG Fc レセプター, IgE レセプター, ヒスタミンレセプタータイプ1上昇を報告した. また G-CSF 投与で EAE 軽症化を認め, イムノグロブリン FcRγ 鎖遺伝子欠損マウスでは EAE 慢性化が抑制されることを報告した[9)].

Mycko ら[10)]は cDNA マイクロアレイ (Clontech)を用いて SPMS 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免疫応答関連遺伝子群 (TNF-α など)の上昇を報告した. Graumann ら[11)]は cDNA マクロアレイ (Clontech)を用いて MS の NAWM における脳虚血関連遺伝子群 (HIF-1α など)の上昇を見出した. Lindberg ら[12)]は GeneChip を用いて SPMS 活動性病巣でのイムノグロブリン産生亢進を見出した. Tajouri ら[13)]は独自の cDNA マイクロアレイを用いて SPMS 急性・慢性活動性病巣における αB-crystallin, SOD1 の上昇を報告した[13)]. これら一連のマイクロアレイによる MS 脳組織の解析は症例数・サンプル数が少なく, RNA 抽出部位が全体像を反映していない可能性が残る.

2) MS とコントロールの末梢血リンパ球の比較解析

Ramanathan ら[14)]は GeneFilter membrane array (Research Genetics)を用いて, MS と健常人の monocyte-depleted PBMC 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. LCK は Airla ら[15)]の cDNA マクロアレイ (Clontech)解析で, RRMS の PBMC において IVMP 治療により低下する遺伝子として報告されている. Bomprezzi ら[16)]は独自の cDNA マイクロアレイを用いて, RRMS と健常人の PBMC で発現差異を呈する 53 遺伝子を同定した. MS では T 細胞活性化関連遺伝子群 IL-7R, ZAP70, TNFRSF7 (CD27)の上昇およびサイトカイン

表 1. MSの免疫病態のマイクロアレイ解析

Authors (Reference No.)	Year	No of MS Patients and Controls	RNA Samples	Type of Microarray	No of Genes on Microarray	Key Findings
Whitney et al[6]	1999	PPMS(n=1)	acute lesion vs NAWM	Original cDNA Glass Array	1,344 or 5,000	Upregulation of IRF-2 and TNFRp75 in acute lesions
Ramanathan et al[14]	2001	RRMS(n=15)vs HC(n=15)	monocyte-depleted PBMC	GeneFilters GF211 Membrane Array(Research Genetics)	5,184	Upregution of LCK, IL-7R and MMP-19 and downregulation of CCR6 and DFFA in MS
Wandinger et al[25]	2001	RRMS(n=1)plus HC(n=2)	PBMC incubated with IFNβ in vitro	Mini-Lymphochip cDNA Array	6,432	Upregulation of proinflammatory genes such as CCR5, IP-10, and IL-15RA by IFN-β treatment
Whitney et al[7]	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	Original cDNA Glass Array	2,798	Upregulation of 5-lipoxygenase in MS and EAE lesions
Lock et al[9]	2002	CPMS and SPMS(n=4)	acute or chronic active lesions vs chronic silent 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-KO mice and by treatment with G-CSF
Mass et al[22]	2002	RA(n=20), SLE(n=24), IDDM(n=5), and MS(n=5)vs HC before and after influenza vaccination(n=9)	PBMC	GeneFilters GF211 Membrane Array(Research Genetics)	4,329	Indistinguishable profiles between MS and IDDM and downregulation of apoptosis-regulatory genes in autoimmune diseases
Bomprezzi et al[16]	2003	RRMS(n=18), SPMS(n=6)vs HC(n=21)	PBMC(fresh or frozen)	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
Graumann et al[11]	2003	SP/PP/RRMS(n=10)vs non-neurological controls(n=7)	NAWM vs control white matter	Atlas Human cDNA Memebrane Array 1.2 (Clontech)	3,528	Upregulation of ischemic preconditioning genes such as HIF-1α in NAWM of MS
Koike et al[26]	2003	RRMS(n=13)before and at 3 and 6 months after IFN-β treatment	T and non-T cells separated from PBMC	Human cDNA Array (Hitachi Life Science)	1,258	Upregulation of 15 IFN-responsive genes in MS after IFN-β treatment
Mycko et al[10]	2003	SPMS(n=4)	chronic active vs silent lesions and the lesion margin vs center	Atlas Human 1.0 Glass Microarray(Clontech)	588	Upregulation of inflammation/immune-related genes in the margin of active lesions

Author	Year	Sample (subjects)	Sample (cells/tissue)	Array	No. of genes	Findings
Stürzbecher et al[34]	2003	RRMS before and after IFN-β treatment for 6 months (n=10, 6 responders vs 4 non-responders)	frozen PBMC ex vivo or incubated with IFN-β in vitro	Mini-Lymphochip cDNA Array	6,432 or 12,672	Downregulation of IL-8 in responders after IFN-β treatment
Tajouri et al[13]	2003	SPMS (n=5) vs non-MS	acute and chronic active lesions	Custom-made cDNA Glass Array	5,000	Upregulation of αB-crystallin and SOD in acute lesions
Weinstock-Guttman et al[32]	2003	RRMS before and at 1, 2, 8, 24, 120, and 160h after IFN-β treatment (n=8)	monocyte-depleted PBMC	GeneFilters GF211 Membrane Array(Research Genetics)	5,184	Time-dependent upregulation of IFN-responsive genes
Achiron et al[19]	2004	RRMS (n=26, 14 with treatment) vs HC (n=18)	PBMC	Human U95Av2 Oligonucleotide Array (Affymetrix)	12,000	Upregulation of T cell activation genes and downregulation of IL-1 and TNF signaling genes in MS
Achiron et al[20]	2004	RRMS treated (n=13) vs untreated (n=13)	PBMC	Human U95Av2 Oligonucleotide Array (Affymetrix)	12,000	Identification of SCYA4, IL2RG, and TNFRSF6(Fas) as immunomodulatory treatment-associated genes
Airla et al[15]	2004	RRMS (n=6) before and after IVMP	PBMC	Atlas Human Hematology/Immunology Membrane Array (Clontech)	448	Downregulation of LCK, TCF7, CD5, and ISGF3 by IVMP
Hong et al[35]	2004	RRMS/SPMS treated with IFN-β (n=18), GA(n=12) or untreated (n=15)	PBMC	Original Membrane Array	36	Distinct gene expression profile between MS patients treated with IFN-β and GA
Iglesias et al[23]	2004	RRMS (n=17) vs HC (n=7)	PBMC	HuGene FL Oligonucleotide Array(Affymetrix)	6,800	Upregulation of E2F transcription factor pathway genes in MS
Lindberg et al[12]	2004	SPMS (n=6) vs non-neurological controls (n=12)	active lesions vs NAWM	Human U95A Oligonucleotide Array (Affymetrix)	12,633	Upregulation of genes related to Ig synthesis in active lesions of MS
Mandel et al[21]	2004	RRMS (n=13) vs SLE (n=5) vs HC (n=18)	PBMC	Human U95Av2 Oligonucleotide Array (Affymetrix)	12,000	Downregulation of NR4A1 and NR4A3 as the autoimmunity-specific signature
Mayne et al[17]	2004	RRMS (n=21) vs HC (n=19)	CD4+T cells	Immune Membrane Array(National Institute on Aging)	1,152	Upregulation of CYFIP2 in MS
Satoh et al[18]	2005	RRMS (n=65) plus SPMS (n=7) vs HC (n=22)	T and non-T cells separated from PBMC	Human cDNA Array (Hitachi Life Science)	1,258	Aberrant expression of apoptosis and DNA damage-regulatory genes in MS

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, IFN : interferon, GA : glatiramer acetate, IVMP : intravenous methylprednisolone pulse

mRNA の分解制御因子 HSPA 1 A(HSP 70)の低下を認めた．Mayne ら[17]は RRMS と健常人の末梢血から CD 4+ T 細胞を negative selection で分離，cDNA membrane array(NIA)を用いて解析し，MS における cytoplasmic FMR 1 interacting protein 2(CYFIP 2)の上昇を報告した．

われわれ[18]は明確な annotation つき 1,259 遺伝子を掲載した cDNA マイクロアレイ(Hitachi Life Science)を用いて，72 例の IFN-β 未治療活動性 MS(65 RRMS，7 SPMS)と 22 名の健常人の末梢血から AutoMACS(Miltenyi Biotec)で分離した CD 3+ T 細胞，CD 3- non-T 細胞の遺伝子発現プロフィールを解析した．両群間で T 細胞 173 遺伝子，non-T 細胞 50 遺伝子の発現差異を認めた．上位 30 遺伝子では T 細胞 25 遺伝子(NR 4 A 2，TCF 8 上昇と MAPK 1，SMARCA 3，HSPA 1 A，TRAIL，TOP 1，CCR 5，BAG 1，DAXX，TSC 22，PARP 低下など)，non-T 細胞 27 遺伝子(ICAM 1，CDC 42，RIPK 2，SODD，TOP 2 A 上昇と BCL 2，RPA 1，NFATC 3，HSPA 1 L，RBBP 4，PRKDC 低下など)がアポトーシス制御遺伝子の範疇に属していた．MS でアポトーシス促進遺伝子(proapoptotic genes)と抑制遺伝子(antiapoptotic genes)の発現上昇・低下(拮抗的バランス counterbalance)を認め，MS におけるアポトーシス制御異常の存在が示唆された．

Achiron ら[19]は GeneChip を用いて 26 例の RRMS と 18 名の健常人で PBMC の遺伝子発現プロフィールを比較解析した．両群間で 1,109 遺伝子の発現差異を認め，MS で T 細胞活性化関連遺伝子群(LEF 1，TCF 3，SLAM，ITGB 2，CTSB)上昇と IL-1β/TNF-α シグナル伝達系遺伝子群の低下を認めた．われわれの結果[18]に反して MS における orphan nuclear receptor NR 4 A 2 の低下を報告した．しかし彼らの研究では MS 14 例は採血時に治療薬 IFN-β，glatiramer acetate(GA)，intravenous immunog-lobulins(IVIg)を投与中で，遺伝子発現に影響した可能性がある．Achiron ら[20]は上記の症例を治療群 13 例と未治療群 13 例に分けて比較し，治療関連 7 遺伝子群(TNFRSF 6，Fas など)を同定した．また 13 例の RRMS と 5 例の SLE を 18 名の健常人と比較し，自己免疫特異的プロフィール(autoimmunity-specific signature)としてアポトーシス制御遺伝子群の発現異常を報告した[21]．Maas ら[22]は 20 例の RA，24 例の SLE，5 例の IDDM，5 例の MS，9 名のインフルエンザワクチン接種前後の健常人の PBMC を解析した．ワクチン免疫応答と自己免疫疾患の遺伝子発現パターンはまったく異なるが，RA と SLE 間，MS と IDDM 間はきわめて類似していた．彼らも自己免疫疾患に共通してアポトーシス制御遺伝子群の発現低下を認めた．Iglesias ら[23]は GeneChip で RRMS と健常人の PBMC を比較解析し，MS における E 2 F transcription factor pathway 遺伝子上昇を認め，E 2 F 1 遺伝子欠損マウスでは EAE が軽症化することを報告した．

3) MS における IFN-β 治療反応性の解析

われわれ[24]は cDNA マクロアレイ(Clontech)を用いて，ヒト胎児脳アストロサイト純培養で IFN により変動する遺伝子を解析し，IFN-β による IRF-7 と pleiotrophin の上昇，IFN-γ による IRF-1 と ICAM-1 の上昇を報告した．Wandinger ら[25]は RRMS と健常人の PBMC を *in vitro* で IFN-β により刺激して cDNA マイクロアレイ(Mini-Lymphochip)で解析し，CC chemokine receptor 5(CCR 5)と interferon-inducible cytokine IP-10(CXCL 10)の上昇を認めた．われわれ[26]は 1,259 遺伝子 cDNA マイクロアレイ(Hitachi)を用いて，13 例の RRMS で IFN-β 治療開始前後に採血し，末梢血 CD 3+ T 細胞と CD 3- non-T 細胞で発現変動した遺伝子群(IFN-responsive genes：IRG)を同定した．T 細胞で 8 遺伝子(IRF-7，ISG 15，IFI 56，IFI 6-

16，IFI 60，IFI 30，ATF 3，TLR 5）の上昇と IL-3，monokine induced by IFN-γ（MIG）の低下，non-T 細胞で 12 遺伝子（IRF-7，ISG 15，IFI 56，IFI 6 - 16，IFI 27，IFI 17，TAP 1，TNFAIP 6，TSC 22，SULT 1 C 1，RPC 39，RAB 11 A）の上昇，IL-3 の低下を認めた．ISG 15，IFI 56，IFI 6-16，IFI 27，TSC 22，SULT 1 C 1 は治療開始後 3～6ヵ月において持続的上昇を認めた．一方統計学的有意差はなかったが，治療後に Th 1 関連遺伝子 CCR 5（T），IFN-γ（T），TNF-α（non-T）の上昇傾向を認めた．この所見は IFN-β 治療は MS では必ずしも明確な Th 2 シフトを誘導しないという見解[25]を支持する．上記の 9 遺伝子（IRF-7，ISG 15，IFI 56，IFI 6-16，IFI 60，IFI 17，TAP 1，TNFAIP 6，MIG）はプロモータ領域に IFN-stimulated response element（ISRE）や IRF element（IRF-E）が同定されている既知 IRG であり，治療に直接反応し治療効果に深く関与していると考えられる．IRF-7 はウイルス感染時に IFN-α/β 産生を増幅する正の制御因子である[27]．IFI 30 はクラス II MHC 拘束性抗原提示の際，はたらくチオール還元酵素で，IFI 30 遺伝子欠損マウスでは抗原呈示能低下をきたす[28]．TAP 1 はクラス I MHC 拘束性抗原提示の際，はたらくペプチド輸送因子で，TAP 1 遺伝子欠損マウスでは CD 8$^+$ T 細胞を介する結核菌抵抗力が減弱する[29]．TNFAIP 6 は TNF-α，IL-1β により誘導される分泌蛋白質で抗炎症作用を呈する[30]．以上のように MS で IFN-β は antiviral and antiinflammatory mediators の発現を誘導することが明らかになった．興味深いことに SLE では治療の種類にかかわらず PBMC における IRG の発現レベルが高い[31]．

Weinstock-Guttman ら[32]は GeneFilter membrane array を用いて，IFN-β 治療前後の 8 例の RRMS で monocyte-depleted PBMC を経時的に解析して IRG を同定した．多くはわれわれの同定した IRG[26]とオーバーラップしている．

Liang ら[33]は Weinstock-Guttman らのデータ[32]を再解析し，IRG は early-onset（8 時間以内），intermediate-onset（24 時間），late-onset（48 時間）の 3 群に分類されることを見出した．Stürzebecher ら[34]は cDNA マイクロアレイ（Mini-Lymphochip）を用いて IFN-β 治療前後の 10 例の RRMS で PBMC の遺伝子発現プロフィールを解析した（ex vivo 解析）．治療前 6ヵ月から開始 12ヵ月後まで毎月 Gd 造影 MRI を撮影して活動性病巣数を算出，治療により病巣数が 60% 以上減少した症例をレスポンダーと定義した．ノンレスポンダー群を当初から効果のみられない nonresponder from initiation of therapy（INR）と，開始後一定期間は効果を認めたが中和抗体（neutralizing antibody：NAb）の出現に伴い効果が減弱した nonresponder with development of NAb（NAbNR）の 2 群に分類した．また培養 PBMC を IFN-β 刺激して in vitro 解析もおこなった．レスポンダーで治療後 2 倍以上変動した遺伝子は ex vivo 25 遺伝子（IFI 17，OAS，Stat 1 上昇と IL-8，CD 69，c-fos，TSC 22 低下など）で，このうち IL-8 発現低下はレスポンダー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，レスポンダー，ノンレスポンダー間で発現差異を認めなかった．彼らの結果と反して，われわれ[26]は IFN-β 治療後の non-T 細胞における TGF-β-stimulated protein TSC 22 上昇を報告している．彼らの研究は症例数が少なく，凍結保存した PBMC を解凍して用いており，実験操作で遺伝子発現が変化しうる点が問題である．また 1 例のレスポンダーでは治療前に約 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 では異例である．Hong ら[35]は主要免疫応答 36 遺伝子を掲載した cDNA マクロアレイを用いて，未治療 MS と IFN-β，GA 治療 MS 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．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
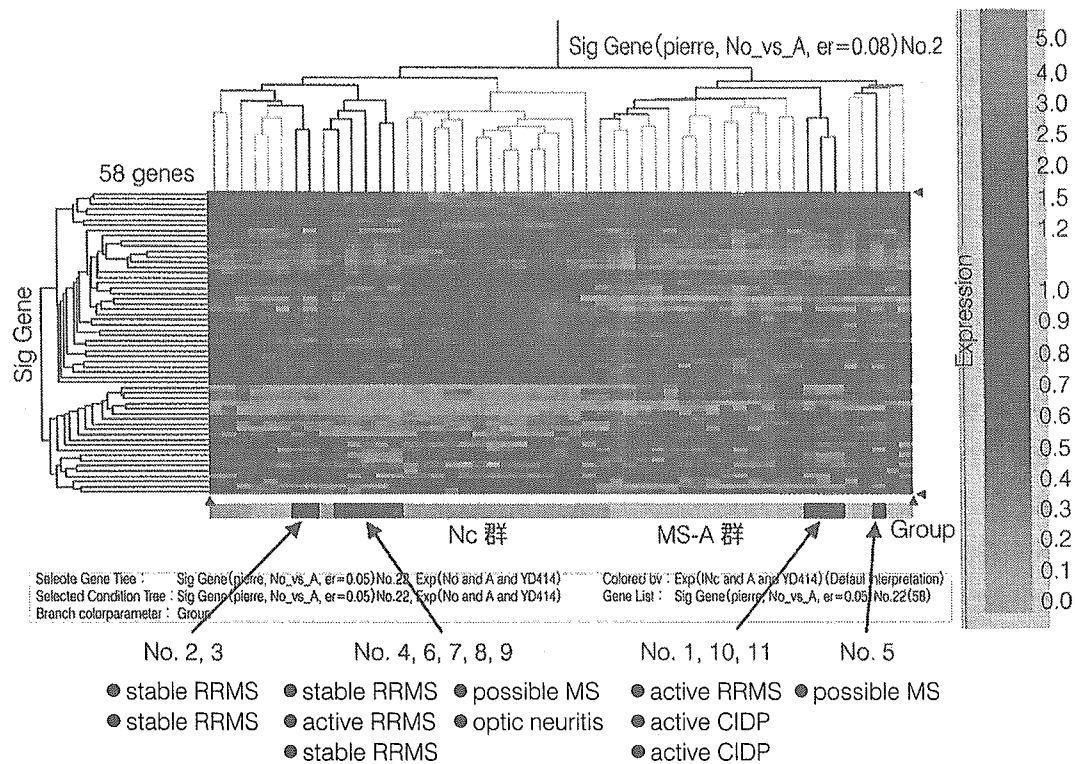
図 1. 階層的クラスター解析

未治療 MS(n＝72)と健常人 Nc(n＝22)の T 細胞の cDNA マイクロアレイ(1,259
遺伝子)解析で発現差異を認める 286 遺伝子を指標遺伝子(discriminator genes)と
する階層的クラスター解析(hierarchical clustering analysis)で，MS 群は Nc 群と
分離され A，B，C，D の 4 群に分類された．A 群は遺伝子発現プロフィールが最も
Nc 群に類似し，群間検定で両者を識別する 58 遺伝子を抽出した．新規 11 症例(2 例
の活動性 MS，4 例の非活動性 MS，2 例の possible MS，1 例の視神経炎，2 例の活
動性慢性炎症性脱髄性多発神経炎)を適合させると，非活動性 MS は全例健常人群
に分類され，一方慢性炎症性脱髄性多発神経炎(chronic inflammatory demyelinat-
ing polyneuropathy：CIDP)は MS-A 群に分類された．

MMP-9 は IFN-β により低下, GA では上昇した．

　van Boxel-Dezire ら[36]は IFN-β 治療を受けた
26 例の RRMS の PBMC におけるサイトカイン
遺伝子発現レベルを半定量的 RT-PCR で経時的
に解析した．治療前後 2 年間の再発回数・IVMP
回数・Extended Disability Status Scale(EDSS)
スコアを集計し比較して 16 例のレスポンダーと
10 例のノンレスポンダーに分けると，レスポン
ダーは治療前に IL-12 p 35 発現レベルが低い傾
向を呈した．Wandinger ら[37]は IFN-β 治療を受
けた RRMS で治療後 1 年間再発がなく EDSS ス
コア悪化のみられない症例をレスポンダー，再発
した症例をノンレスポンダーと定義した．20 例の

レスポンダーと 19 例のノンレスポンダーを比較
すると，レスポンダーでは TNF-related
apoptosis-inducing ligand(TRAIL, TNFSF 10)
が持続的高値を示すことを見出した．TRAIL は
IRG の 1 つで，われわれ[18]は MS T 細胞における
発現低下を報告している．TRAIL 遺伝子欠損マ
ウスは胸腺細胞アポトーシスに異常をきたし，コ
ラーゲン関節炎に高感受性になる[38]．Baranzini
ら[39]は IFN-β 治療を受けた 52 例の RRMS で
PBMC における 70 遺伝子の発現レベルを経時的
に定量的 RT-PCR で解析した．治療後 2 年間 1
度も再発がなく EDSS スコア悪化のない症例を
レスポンダー，2 回以上再発した症例をノンレス

表 2. SVM 解析によるグループ分類

					Case No.	1	2	3	4	5	6	7	8	9	10	11
					Age/Sex	40 F	57 F	30 M	37 M	36 F	48 F	61 F	37 F	22 F	16 M	18 M
					Clinical Diagnosis	Active RRMS	Stable RRMS	Stable RRMS	Stable RRMS	Possible MS	Active RRMS	Stable RRMS	Possible MS	Optic Neuritis	CIDP	CIDP
					Clustering by 58 genes	A	Nc	Nc	Nc	A	Nc	Nc	Nc	Nc	A	A
SVM No.	Gene Set	Gene Selection Method	No. of Predictor Genes	Kernel Function		SVM Classification										
1	58	All genes	—	PDP(Order 1)		A	Nc	A	A	A	Nc	A	Nc	Nc	A	A
2	58	All genes	—	PDP(Order 2)		A	Nc	A	A	A	Nc	Nc	Nc	Nc	A	A
3	58	All genes	—	PDP(Order 3)		A	Nc	A	A	A	Nc	Nc	Nc	Nc	A	A
4	58	All genes	—	Radial basis(Gaussian)		A	Nc	A	A	A	Nc	Nc	Nc	Nc	A	A
5	286	All genes	—	PDP(Order 1)		A	Nc	A	A	A	Nc	Nc	Nc	Nc	A	A
6	286	All genes	—	PDP(Order 2)		A	Nc	A	A	A	Nc	Nc	Nc	Nc	A	A
7	286	All genes	—	PDP(Order 3)		A	Nc	A	A	A	Nc	Nc	Nc	Nc	A	A
8	286	All genes	—	Radial basis(Gaussian)		A	Nc	A	A	A	Nc	Nc	Nc	Nc	A	A
9	286	Fisher's Exact Test	50	PDP(Order 1)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	A
10	286	Fisher's Exact Test	50	PDP(Order 2)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	A
11	286	Fisher's Exact Test	50	PDP(Order 3)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	A
12	286	Fisher's Exact Test	50	Radial basis(Gaussian)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	A
13	286	Golub Method	50	PDP(Order 1)		A	Nc	A	A	A	Nc	Nc	Nc	Nc	A	A
14	286	Golub Method	50	PDP(Order 2)		A	Nc	A	A	A	Nc	Nc	Nc	Nc	A	A
15	286	Golub Method	50	PDP(Order 3)		A	Nc	A	A	A	Nc	Nc	Nc	Nc	A	A
16	286	Golub Method	50	Radial basis(Gaussian)		A	Nc	A	A	A	Nc	Nc	Nc	Nc	A	A
17	286	Fisher's Exact Test	30	PDP(Order 1)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	A
18	286	Fisher's Exact Test	30	PDP(Order 2)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	A
19	286	Fisher's Exact Test	30	PDP(Order 3)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	A
20	286	Fisher's Exact Test	30	Radial basis(Gaussian)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	A
21	286	Golub Method	30	PDP(Order 1)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	Nc
22	286	Golub Method	30	PDP(Order 2)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	Nc
23	286	Golub Method	30	PDP(Order 3)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	Nc
24	286	Golub Method	30	Radial basis(Gaussian)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	Nc
25	286	Fisher's Exact Test	10	PDP(Order 1)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	A
26	286	Fisher's Exact Test	10	PDP(Order 2)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	A
27	286	Fisher's Exact Test	10	PDP(Order 3)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	Nc	Nc
28	286	Fisher's Exact Test	10	Radial basis(Gaussian)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	Nc	Nc
29	286	Golub Method	10	PDP(Order 1)		A	Nc	A	Nc	A	Nc	Nc	A	Nc	A	Nc
30	286	Golub Method	10	PDP(Order 2)		A	Nc	A	Nc	A	Nc	Nc	A	Nc	A	Nc
31	286	Golub Method	10	PDP(Order 3)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	Nc
32	286	Golub Method	10	Radial basis(Gaussian)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	Nc
33	1259	All genes	—	PDP(Order 1)		A	A	A	A	A	A	Nc	Nc	Nc	A	A
34	1259	All genes	—	PDP(Order 2)		A	A	A	A	A	Nc	Nc	Nc	A	A	A
35	1259	All genes	—	PDP(Order 3)		A	A	A	A	A	Nc	Nc	A	Nc	A	A
36	1259	All genes	—	Radial basis(Gaussian)		A	A	A	A	A	Nc	Nc	Nc	A	A	A
37	1259	Fisher's Exact Test	50	PDP(Order 1)		A	Nc	A	A	A	Nc	Nc	Nc	Nc	A	A
38	1259	Fisher's Exact Test	50	PDP(Order 2)		A	Nc	A	A	A	Nc	Nc	Nc	Nc	A	A
39	1259	Fisher's Exact Test	50	PDP(Order 3)		A	Nc	A	A	A	Nc	Nc	Nc	Nc	A	A
40	1259	Fisher's Exact Test	50	Radial basis(Gaussian)		A	Nc	A	A	A	Nc	Nc	Nc	Nc	A	A
41	1259	Golub Method	50	PDP(Order 1)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	A
42	1259	Golub Method	50	PDP(Order 2)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	A
43	1259	Golub Method	50	PDP(Order 3)		A	Nc	A	A	A	Nc	Nc	Nc	Nc	A	A
44	1259	Golub Method	50	Radial basis(Gaussian)		A	Nc	A	A	A	Nc	Nc	Nc	Nc	A	A
45	1259	Fisher's Exact Test	30	PDP(Order 1)		A	Nc	A	A	A	Nc	Nc	Nc	Nc	A	A
46	1259	Fisher's Exact Test	30	PDP(Order 2)		A	Nc	A	A	A	Nc	Nc	Nc	Nc	A	A
47	1259	Fisher's Exact Test	30	PDP(Order 3)		A	Nc	A	A	A	Nc	Nc	Nc	Nc	A	A
48	1259	Fisher's Exact Test	30	Radial basis(Gaussian)		A	Nc	A	A	A	Nc	Nc	Nc	Nc	A	A
49	1259	Golub Method	30	PDP(Order 1)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	A
50	1259	Golub Method	30	PDP(Order 2)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	A
51	1259	Golub Method	30	PDP(Order 3)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	A
52	1259	Golub Method	30	Radial basis(Gaussian)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	A
53	1259	Fisher's Exact Test	10	PDP(Order 1)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	A
54	1259	Fisher's Exact Test	10	PDP(Order 2)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	A
55	1259	Fisher's Exact Test	10	PDP(Order 3)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	A
56	1259	Fisher's Exact Test	10	Radial basis(Gaussian)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	A
57	1259	Golub Method	10	PDP(Order 1)		Nc	Nc	Nc	Nc	Nc	Nc	Nc	Nc	Nc	A	Nc
58	1259	Golub Method	10	PDP(Order 2)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	Nc
59	1259	Golub Method	10	PDP(Order 3)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	Nc
60	1259	Golub Method	10	Radial basis(Gaussian)		A	Nc	A	Nc	A	Nc	Nc	Nc	Nc	A	A

未治療 MS(n=72)と健常人 Nc(n=22)の T 細胞の cDNA マイクロアレイ(1,259 遺伝子)解析で発現差異を認める 286 遺伝子を指標遺伝子(discriminator genes)とする階層的クラスター解析(hierarchical clustering analysis)で、MS 群は Nc 群と分離され A, B, C, D の 4 群に分類された. A 群は遺伝子発現プロフィールが最も Nc 群に類似し, 群間検定で両者を識別する 58 遺伝子を抽出した. Gene Set：SVM 実行に使用された遺伝子セット, Gene Selection Method：predictor genes を選定した検定法, Kernel Function：グループ・クラス判別に使用された Kernel 関数の種類

ポンダーと定義し，両者は3遺伝子［caspase 2, caspase 10, FLICE inhibitory protein (FLIP)］の発現レベルの三次元マッピングで86%区別可能と報告した．

最近，われわれ[18]は72例のIFN-β未治療活性MS（46例は初回採血後2年間IFN-β治療開始）と22名の健常人の末梢血CD 3+ T細胞を1,259遺伝子cDNAマイクロアレイ (Hitachi)で解析したデータに関して，両群間で発現差異を示す286遺伝子を指標遺伝子 (discriminator genes)にして階層的クラスター解析を施行した［MS classification database (MSCD), Satoh et al, Manuscript in preparation］．286遺伝子は5クラス（クラス#1〜#5）に分類され，MS群は健常人群から分離され，さらに4グループ（A，B，C，D）に分類された．A群は遺伝子発現プロフィールが最も健常人に類似し，B群は最も臨床的活性が高く，ケモカイン遺伝子の多いクラス#5の発現レベルが高く，C群は大脳限局病変を呈する患者が多く，D群は最もEDSSスコアが高値であった．さらに群間検定で健常人とMS A群を識別する58遺伝子を抽出した．治療前後2年間の再発回数・IVMP日数・入院日数・EDSSスコア・MRI T 2強調画像病巣数と患者満足度から治療評価スコアを算出すると，レスポンダーはA群とB群に集積していた．レスポンダーではノンレスポンダーに比較して，治療開始後6ヵ月のIRG (ISG 15, IFI 27, MCP-1, TNFRp 75)発現レベルが高く維持されていた．

このMSCDに新規11症例（2例の活性MS，4例の非活性MS，2例のpossible MS，1例の視神経炎，2例の活性慢性炎症性脱髄性多発神経炎）を適合させると，286遺伝子，58遺伝子を指標遺伝子とする階層的クラスター解析により，非活性MSは全例健常者群に分類された（図1）．一方慢性炎症性脱髄性多発神経炎 (chronic inflammatory demyelinating polyneuropathy：CIDP)はMS A群に分類された．MSCDを

training setとして施行したSVM解析でも同様のグループ分類が支持された（表2）．症例数は少ないがわれわれの結果は末梢神経抗原に対する自己免疫機序が関与するCIDPと中枢神経抗原に対する自己免疫機序が関与するMSではT細胞遺伝子発現レベルで類似性があり，CIDPはDNAマイクロアレイ解析だけではMSと鑑別できないことを示唆する．

おわりに

遺伝子アレイ解析は臨床所見や画像のみでは鑑別困難な疾患の補助診断のツール，腫瘍悪性度や予後の予測，薬物反応性や副作用の予測，治療効果の判定など幅広い臨床応用が期待されている．われわれはDNAマイクロアレイ解析でMSがT細胞遺伝子発現プロフィールにもとづき4群に分類され，各群は疾患活性・病変分布・IFN-β治療反応性と密接な対応を認めることを報告した（MS classification database：MSCD）．今後MSCDを礎にしてテーラメイド医療樹立に向けて研究を進める予定である．

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Preferential T$_h$2 polarization by OCH is supported by incompetent NKT cell induction of CD40L and following production of inflammatory cytokines by bystander cells *in vivo*

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Abstract

The altered glycolipid ligand OCH is a selective inducer of T$_h$2 cytokines from NKT cells and a potent therapeutic reagent for T$_h$1-mediated autoimmune diseases. Although we have previously shown the intrinsic molecular mechanism of preferential IL-4 production by OCH-stimulated NKT cells, little is known about the extrinsic regulatory network for IFN-γ production. Here we demonstrate that OCH induces lower production of IFN-γ, not only by NKT cells but also by NK cells compared with α-galactosylceramide. OCH induced lower IL-12 production due to ineffective primary IFN-γ and CD40 ligand expression by NKT cells, and resulted in lower secondary IFN-γ induction. Co-injection of a sub-optimal dose of IFN-γ and stimulatory anti-CD40 mAb compensates for the lower induction of IL-12 by OCH administration. IL-12 converts OCH-induced cytokine expression from IL-4 predominance to IFN-γ predominance. Furthermore, CpG oligodeoxynucleotide augmented IL-12 production when co-administrated with OCH, resulting in increased IFN-γ production. Taken together, the lower IL-12 production and subsequent lack of secondary IFN-γ burst support the effective T$_h$2 polarization of T cells by OCH. In addition, highlighted in this study is the characteristic property of OCH that can induce the differential production of IFN-γ or IL-4 according to the availability of IL-12.

Introduction

NKT cells are a unique subset of CD1d-restricted T lymphocytes that express TCR and some NKR. NKT cells recognize glycolipid antigens such as α-galactosylceramide (αGC) by an invariant TCRα chain composed of Vα14-Jα18 segments in mice and Vα24-Jα18 segments in humans, associated with TCRβ chains using a restricted set of Vβ genes (1, 2). NKT cells rapidly secrete large amounts of cytokines including IL-4 and IFN-γ upon antigen stimulation and are effective regulators of T$_h$1/T$_h$2 balance *in vivo* (3–5). We have previously demonstrated that *in vivo* administration to mice of altered glycolipid ligand, OCH, ameliorates experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA) and type I diabetes by enhancing IL-4-dependent T$_h$2 responses without inducing IFN-γ production and pathogenic T$_h$1 responses (6–8).

Recently, we have clarified the intrinsic molecular mechanism of preferential IL-4 production by OCH-stimulated NKT cells (9). IFN-γ production by NKT cells was more susceptible

to the sphingosine length of glycolipid ligand than that of IL-4, and the length of sphingosine chain determined the half-life of NKT cell stimulation by CD1d-associated glycolipids. IFN-γ production by NKT cells required longer T cell stimulation than did IL-4 production and the transcription of the IFN-γ gene required *de novo* protein synthesis by activated NKT cells. The NF-κB family member transcription factor c-Rel was preferentially transcribed in αGC-stimulated, but not in OCH-stimulated, NKT cells and was identified as essential for IFN-γ production by activated NKT cells. Therefore, the differential duration of NKT cell stimulation, due to the binding stability of individual glycolipid antigens to CD1d molecules, determines whether signaling leads to effective c-Rel transcription and IFN-γ production by activated NKT cells.

Upon stimulation by αGC *in vivo*, NKT cells rapidly affect the functions of neighboring cell populations such as T cells, NK cells, B cells and dendritic cells (DCs) in a direct or indirect manner (10–13). The serial production of IFN-γ by NKT cells

and NK cells has been demonstrated, suggesting that activated NKT cells may influence further IFN-γ production by other cells including NK cells (3, 10). A C-glycoside analog of αGC has been shown to induce a superior T$_h$1-type response than αGC does by inducing higher IFN-γ production by NK cells. IL-12 was indispensable for the T$_h$1-skewing effect of the glycolipid, indicating the importance of IL-12 in enhanced IFN-γ production *in vivo* (14). Furthermore, αGC-stimulated NKT cells can act as an adjuvant *in vivo* by inducing the full maturation of DCs, as manifested by augmented co-stimulatory molecules and enhanced mixed leukocyte reactions (11). Accordingly, αGC-stimulated NKT cells were shown to express CD40 ligand (CD40L, CD154), which can engage CD40 on antigen-presenting cells and stimulate them to produce IL-12 (15, 16). Furthermore, IFN-γ production and T$_h$1-type responses were impaired in CD40-deficient mice (5). A growing body of evidence suggests that both extrinsic and intrinsic factors compose an intricate network for controlling IFN-γ production and T$_h$1 polarization after intensive stimulation of NKT cells by superagonistic glycolipid such as αGC.

Although the intrinsic molecular mechanism of preferential IL-4 production by OCH-stimulated NKT cells has been elucidated, little is known about the effect of OCH on bystander cells and the extrinsic regulatory network for IFN-γ production and T$_h$1 polarization. Considering the lower IFN-γ production by OCH compared with extensive IFN-γ production by αGC *in vivo*, OCH may affect the functions of neighboring cell populations in a different manner from that of αGC. In the current study, we demonstrate that OCH induces less effective production of IFN-γ and IL-12 by bystander cells possibly due to lower expression of CD40L by NKT cells. Co-administration of stimulatory anti-CD40 mAb in combination with IFN-γ enhanced the production of IL-12 induced by OCH *in vivo*, and IL-12 modulated OCH-induced cytokine expression by augmenting IFN-γ. Consistent with these results, co-administration of CpG oligodeoxynucleotide (ODN) with OCH preferentially induced IFN-γ production possibly through augmented IL-12 production. Considering that NKT cell responses to CD1d-presented self-antigens are modified by IL-12 to induce massive IFN-γ production during the course of microbial infection (17), OCH, at least partly, mimics the physiological behavior of the putative self-antigen for NKT cells in the context of cytokine milieu *in vivo*.

Methods

Reagents and antibodies

Murine IL-12, IFN-γ and Flt3-ligand (Flt3L) were purchased from Peprotech EC (London, UK). Anti-CD40 mAb (HM40-3) was purchased from BD Biosciences PharMingen (San Diego, CA, USA). Mouse anti-IFN-γ (R4-6A2) was purified from ascites of hybridoma obtained from American Type Culture Collection. Glycolipids were solubilized in dimethyl sulfoxide (100 μg ml^{-1}) and stored at −20°C until use. The following CpG ODN was synthesized: CpG ODN, 5′-GCATGACGTT-GAGCT-3′.

Mice

C57BL/6 (B6) mice were purchased from CLEA Laboratory Animal Corporation (Tokyo, Japan). MHC class II-deficient I-A^{b}β−/− mice with the B6 background were purchased from Taconic (Germantown, NY, USA). All animals were kept under specific pathogen-free conditions and used at 7–12 weeks of age. Animal care and use were in accordance with institutional guidelines.

Induction of bone marrow-derived DCs

Bone marrow cells were isolated by flushing femurs of B6 mice and re-suspended in culture medium supplemented with murine Flt3L (100 ng ml^{-1}) as described in (18). Cells were harvested from the culture after 10 days and subjected to co-culture experiment with NKT cells.

Flow cytometry and intracellular cytokine staining

Spleen cells or liver mononuclear cells harvested after stimulation with glycolipids *in vivo* were cultured in complete media containing GolgiStop (BD PharMingen, San Jose, CA, USA). Then cells were incubated with Fc block (anti-mouse FcγIIIR/IIR mAb clone 2.4G2) and were stained with biotinylated anti-NK1.1 mAb (PK136), washed with PBS and then stained with peridinin chlorophyll protein/cyanine 5.5–anti-CD3 mAb and streptavidin–allophycoerythrin (APC). Then cells were washed twice with PBS and fixed in BD Cytofix/Cytoperm solution for 20 min at 4°C. After fixation, cells were washed with BD Perm/Wash solution and re-suspended in the same solution containing either PE–anti-IFN-γ mAb (XMG1.2) or PE-conjugated isotype control Ig for 30 min at 4°C. Then samples were washed and the stained cells were analyzed using a FACS Calibur instrument (Becton Dickinson) with CELLQuest software (Becton Dickinson). Identification of iNKT cells by Dimer XI Recombinant Soluble Dimeric Mouse CD1d (BD PharMingen) was performed as described previously (19). For analysis of CD40L expression, spleen cells harvested after stimulation with glycolipids *in vivo* for indicated periods of time were cultured in complete media containing biotinylated anti-CD40L mAb (MR1) for 2 h. Cells were harvested, washed with PBS and stained with FITC–anti-CD3 mAb, PE–anti-NK1.1 mAb and streptavidin–APC for 20 min. CD40L expression was analyzed in CD3/NK1.1 double-positive cell.

Microarray

Microarray analysis was performed as described previously (9). In brief, I-A^{b}β−/− mice pre-treated with anti-asialo GM$_1$ antibody were injected with αGC or OCH (100 μg kg^{-1}). Total RNA was isolated from liver NKT cells (purified as CD3+ NK1.1+ cells) and applied to microarray by using U74Av2 arrays (GeneChip System, Affymetrix, Santa Clara, CA, USA). From data image files, gene transcript levels were determined using algorithms in the GeneChip Analysis Suit software (Affymetrix).

Quantitative reverse transcription–PCR

Quantitative reverse transcription–PCR was conducted using a Light Cycler-FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals) as described previously (9). Primers used for the analysis of gene expression are as follows; CD40L (F) CGAGTCAACGCCCATTCATC, (R) GTAATTCAAA-CACTCCGCCC.

ELISA

The level of cytokine production in cell culture supernatants or in serum was evaluated by standard sandwich ELISA, employing purified and biotinylated mAb sets (11B11/BVD6-24G2 for IL-4, R4-6A2/XMG1.2 for IFN-γ and 9A5/C17.8 for IL-12) and standards (OptEIA set, BD PharMingen) as described previously (9). After adding a substrate, the reaction was evaluated using a Microplate reader (BioRad).

Statistics

For statistic analysis, non-parametric Mann–Whitney test was used to calculate significance levels for all measurements. Values of $P < 0.05$ was considered statistically significant.

Results

OCH induces lower IFN-γ expression than αGC in both NKT cells and NK cells in vivo

Although NKT cells are a major source of IL-4 after glycolipid administration *in vivo*, activated NKT cells are shown to affect the functions of bystander cells such as T cells, NK cells, B cells and DCs in a direct or indirect manner, resulting in

possible secondary augmentation of IFN-γ production by these cells. To evaluate the contribution of NKT cells and other cells for IFN-γ production after glycolipid administration, we performed kinetic analysis of cytokine production by splenic NKT cells, NK cells, T cells and other cells after *in vivo* administration of glycolipids. IFN-γ production was detected both in NKT cells and NK cells (Fig. 1A), and neither CD3+ T cells nor CD3−NK1.1− cells showed significant IFN-γ production 2 or 6 h after glycolipid administration. αGC induced a larger population of IFN-γ-producing NKT cells than OCH did which is consistent with the previous report (9). The kinetic analysis revealed that IFN-γ production by NKT cells was dominant in earlier time points (2 h) after glycolipid administration and IFN-γ production by NK cells was comparable or even higher at later time points (6 h) (Fig. 1B), suggesting that IFN-γ production by NKT cells preceded IFN-γ production by NK cells as reported previously (3, 10). As CD3+NK1.1+ cells do not always represent CD1d-restricted iNKT cells, we compared IFN-γ production by CD1d-dimerX-positive T cells after treatment with αGC or OCH. Again, αGC induced a larger population of IFN-γ-producing iNKT cells than OCH did (Fig. 1C). Interestingly, αGC induced a much larger population of IFN-γ-producing NK cells than
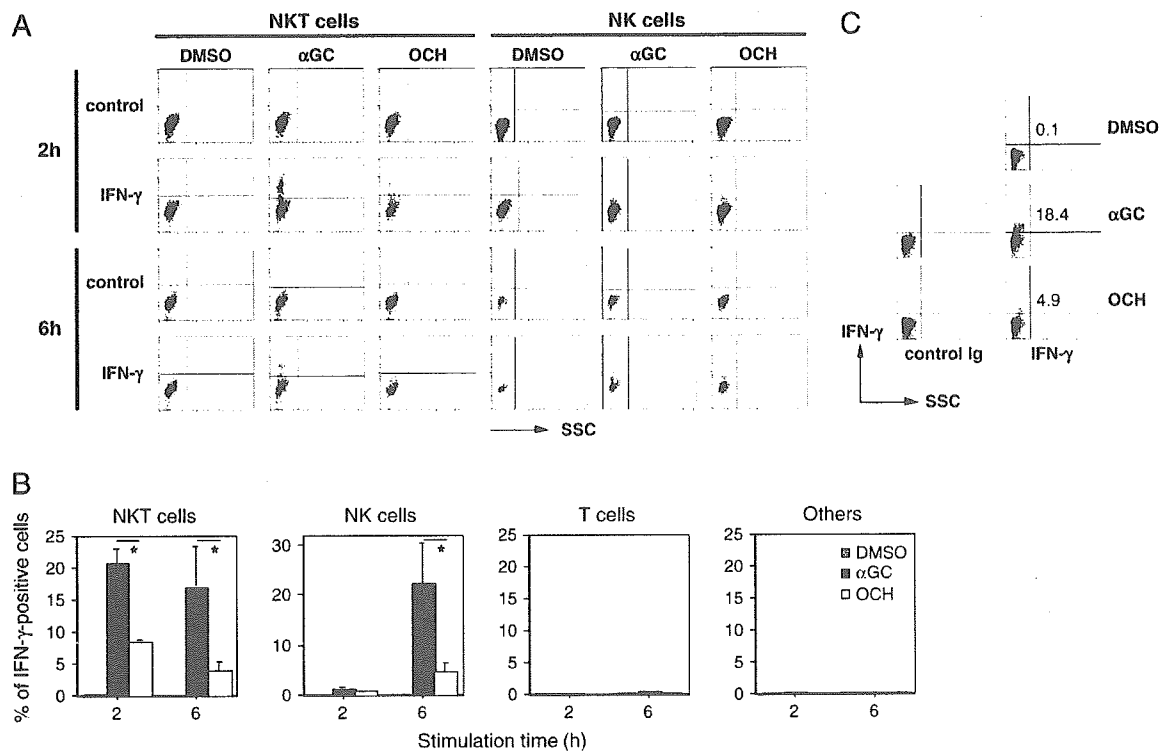

Fig. 1. Expression of IFN-γ by NKT cells and non-NKT cells after administration of glycolipid ligands. B6 mice were treated intra-peritoneally with 2 μg per mouse of either αGC or OCH, and spleen cells were harvested at various time points after glycolipid administration and subjected to intracellular cytokine staining as described in Methods. (A) Data analyzed for CD3+NK1.1+ NKT cells, CD3−NK1.1+ NK cells, CD3+NK1.1− T cells and CD3−NK1.1− cells were shown for the presence of intracellular IFN-γ. Similar results were obtained by analyzing liver mononuclear cells after glycolipid administration (data not shown). (B) Plotted values represent the percentage of IFN-γ-positive cells (mean ± SD for triplicate samples) in the gated population after treatment with dimethyl sulfoxide (DMSO) (hatched bar), αGC (filled bar), or OCH (open bar). (C) Data analyzed for CD1d-DimerX-positive iNKT cells were shown for the presence of intracellular IFN-γ 2 h after glycolipid treatment. The experiments shown are representative of three independent experiments. *$P < 0.05$.

OCH, suggesting that OCH induces less IFN-γ production than αGC not only by direct effect on NKT cells but also by indirect effect on NK cells. To exclude the possibility of the contamination of activated non-CD1d-restricted T cells into NKT fractions or activated NKT cells into NK cells fraction due to the down-regulation of TCR, we conducted the following experiments. First, αGC-loaded DimerXl-stained cells were concentrated in the NK1.1+CD3+ population and <0.4% of cells were reactive to αGC-loaded DimerXl either in NK1.1+CD3− or NK1.1−CD3+ cell populations. Second, >95% of αGC-loaded DimerXl-reactive spleen cells were positive for both CD3 and NK1.1 after stimulation with glycolipids. Third, most of the intracellular IFN-γ-positive CD3− cells were DX5 positive 2 and 6 h after stimulation with glycolipids (data not shown). These results indicated that the contamination of IFN-γ-producing cells into the other fractions was minimum.

αGC-induced IFN-γ production by NK cells is partly dependent on IFN-γ produced by NKT cells

To determine the effect of IFN-γ on consequent IFN-γ production by NK cells, we treated mice with anti-IFN-γ mAb before administration of αGC, and then examined IFN-γ-producing cells using intracellular staining. As shown in Fig. 2, there was no significant difference in the frequency of IFN-γ-producing NKT cells after administration of αGC with or without anti-IFN-γ mAb. Meanwhile, co-administration of anti-IFN-γ mAb showed ~35% reduction in IFN-γ-producing NK cells after αGC treatment (Fig. 2, right panel). These results suggested that NKT cell-derived IFN-γ was involved in αGC-induced IFN-γ production by NK cells to some extent, but an IFN-γ-independent mechanism might be involved in indirect up-regulation of IFN-γ production by NK cells after αGC administration *in vivo*.
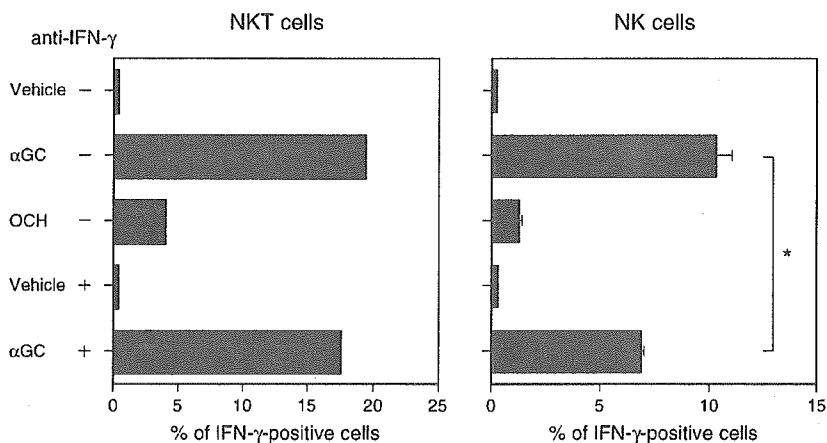
OCH administration does not induce effective IL-12 production

As DCs were demonstrated to be activated after *in vivo* administration of αGC (11, 20) to produce large amount of IL-12 (21) and IL-12 is one of the most potent inducers of IFN-γ (22), we performed kinetic cytokine analysis of serum levels of IL-12 (p70) together with IFN-γ and IL-4 after intra-peritoneal injection of the glycolipids into B6 mice. As shown in Fig. 3, administration of αGC induced a rapid elevation of IL-4 and a delayed elevation of IFN-γ in B6 mice. In contrast, administration of OCH induced a rapid elevation of IL-4 comparable to that induced by αGC with significantly less amount of elevation of IFN-γ, resulted in T_h2 skewing as described previously. Although the level of IL-12 in serum was observed 6 h after αGC injection, OCH injection induced one-tenth amount of serum IL-12 level compared with αGC. In addition, freshly isolated liver NKT cells co-cultured with Flt3L-induced DCs produced significantly higher amount of IL-12 in the presence of αGC compared with OCH. Meanwhile, Flt3L-induced DCs loaded with either αGC or OCH exerted comparable amount of IL-4 production (Fig. 3B), demonstrating directly that DCs loaded with OCH produce less IL-12 upon co-culture with NKT cells than DCs loaded with αGC, and therefore suggest that the *in vivo* effects of OCH are not simply due to its preferential presentation by antigen-presenting cells that produce less IL-12. Taken together, these results indicated that OCH administration did not induce effective IL-12 production *in vivo*.

Lower expression of CD40L on OCH-stimulated NKT cells

Activated NKT cells stimulate DCs to produce IL-12 through the engagement of CD40 on DCs with CD40L inducibly expressed on NKT cells (15, 21). Furthermore, a C-glycoside analog of αGC induced a superior IFN-γ production by NK cells than αGC does in an IL-12-dependent manner (14),
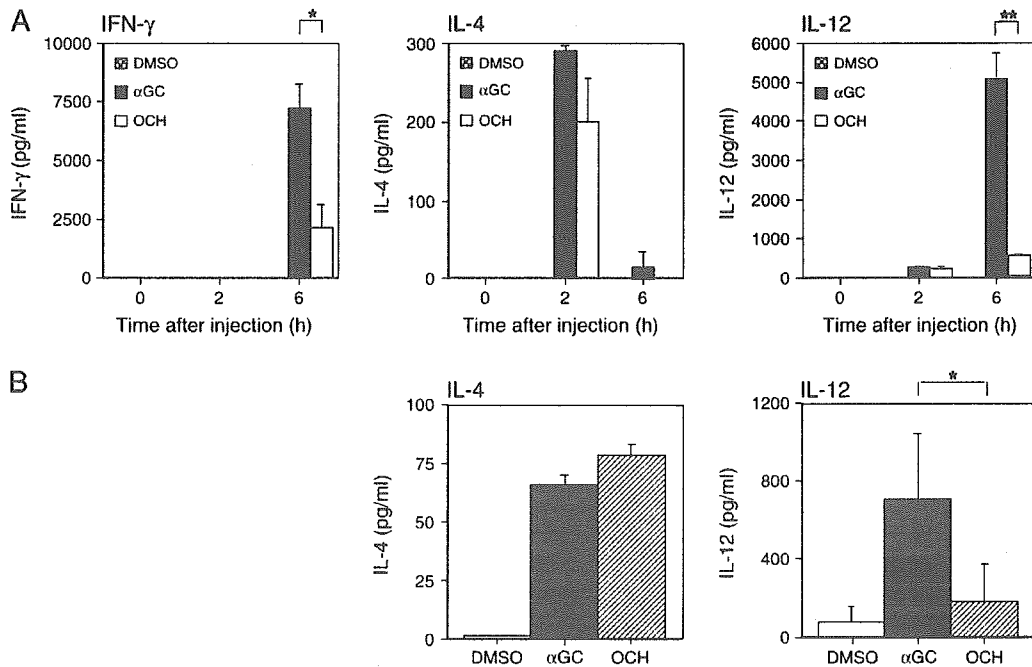

Fig. 2. αGC-induced IFN-γ production by NK cells is partly dependent on IFN-γ production by NKT cells. B6 mice were treated intra-peritoneally with 2 μg per mouse of glycolipids with or without 500 μg per mouse of anti-IFN-γ mAb. Four hours after treatment, spleen cells were harvested and subjected to intracellular cytokine staining. Plotted values represent the percentage of IFN-γ-positive cells (mean ± SD for triplicate samples) in the gated population for CD3+NK1.1+ NKT cells (left) or CD3−NK1.1+ NK cells (right). Similar results were obtained by analyzing liver mononuclear cells after glycolipid administration (data not shown). The experiments shown are representative of three independent experiments. *P < 0.05.

Fig. 3. OCH administration does not induce effective IL-12 production. (A) B6 mice were injected intra-peritoneally with vehicle alone, 2 μg per mouse of αGC or OCH and serum samples were collected at indicated times after injection. Serum levels of IFN-γ, IL-4 and IL-12 (mean ± SD) were determined by ELISA. This figure represents one of two experiments with similar results. *$P < 0.05$, **$P < 0.01$. (B) Freshly isolated liver NKT cells were co-cultured with Flt3L-induced DCs in the presence of αGC or OCH for 72 h. Levels of IL-4 and IL-12 were determined by ELISA. Data are expressed as mean ± SD for triplicate wells and representative data of two similar experiments are shown. *$P < 0.05$.

which suggests that IFN-γ production by NK cells might be regulated by IL-12. To clarify the mechanisms of lack of IL-12 production upon stimulation with OCH, we compared the inducible expression of CD40L on NKT cells after *in vivo* administration of glycolipids. Microarray analysis revealed that CD40L transcripts were inducibly expressed in NKT cells 1.5 h after stimulation with αGC and disappeared 12 h after stimulation. In contrast, OCH treatment induced approximately one-third of CD40L transcription compared with the effect of αGC (Fig. 4A). Consistent with the data of microarray analysis, real-time PCR analysis confirmed the preferential up-regulation of CD40L transcript after αGC stimulation (Fig. 4B). To demonstrate the differential expression of CD40L between αGC-stimulated and OCH-stimulated NKT cells, surface expression of CD40L on NKT cells were compared by flow cytometry after *in vivo* treatment with the glycolipids. As shown in Fig. 4(C), αGC induced higher expression of CD40L than OCH did on the surface of NKT cells. If compared quantitatively by mean fluorescence intensity of CD40L-positive subsets after treatment with either glycolipid, OCH treatment induced less CD40L expression on NKT cells compared with the effect of αGC (Fig. 4C, right panel). These results indicated that CD40L expression on αGC-stimulated NKT cells was significantly higher than that on OCH-stimulated NKT cells.

Co-administration of IFN-γ and CD40 stimulation augments IL-12 production by OCH in vivo

Although the CD40 pathway plays an intrinsic role in physiological conditions in eliciting IL-12 production, effective production of bioactive IL-12 by DCs requires another signal mediated by innate signals such as microbial stimuli (23) or by IFN-γ (24–26). Therefore, OCH-induced expression of CD40L and IFN-γ may not be effective to initiate IL-12 production from DCs *in vivo*. To test this hypothesis, we examined whether co-administration of stimulatory anti-CD40 mAb and/or IFN-γ confer OCH to induce higher IL-12 production. As shown in Fig. 5, administration of IFN-γ, stimulatory anti-CD40 mAb or combination of both reagents did not induce IL-12 expression *in vivo*. On the contrary, OCH-induced IL-12 production was partially augmented by co-administration of anti-CD40 mAb. Furthermore, concomitant administration of IFN-γ and stimulatory anti-CD40 mAb with OCH induced IL-12 production. These results suggest that the signals through CD40 and IFN-γ provided by OCH-stimulated NKT cells did not lead to efficient production of IL-12.

Co-administration of IL-12 augments IFN-γ production by OCH in vivo

A series of experiments so far indicated that OCH was less effective for induction of CD40L, IFN-γ and consequent IL-12 production than those induced by αGC. To examine directly the role of IL-12 production in less effective IFN-γ production by NKT cells and NK cells after OCH administration, we tested whether co-administration of IL-12 with OCH induces IFN-γ *in vitro* and *in vivo*. As shown in Fig. 6(A), IL-12 augmented IFN-γ production from spleen cells after *in vitro* treatment with
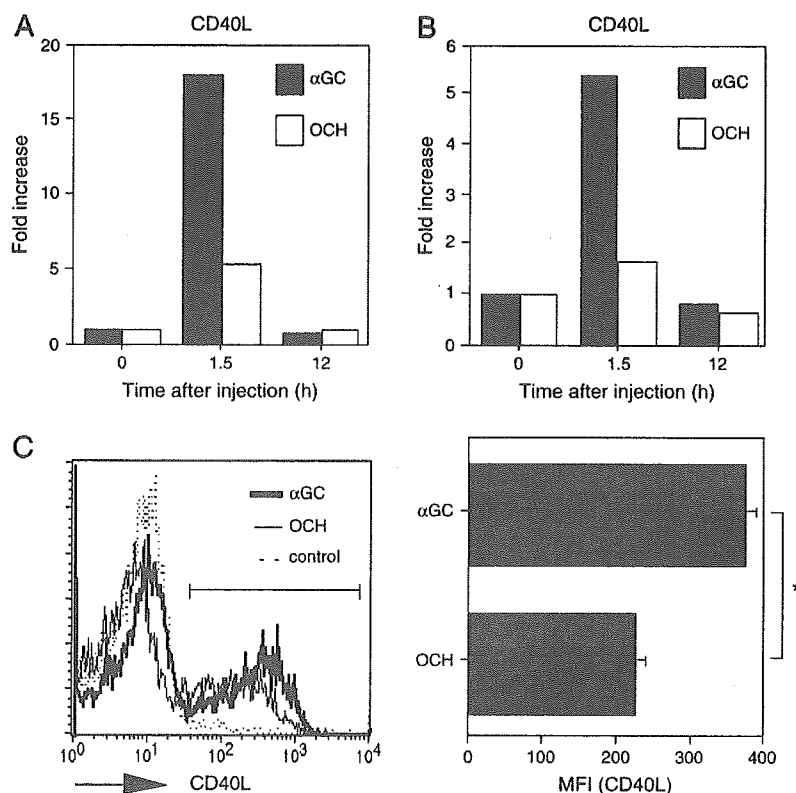
Fig. 4. Expression of CD40L on NKT cells stimulated with αGC or OCH. (A) Plotted values represent data of Affymetrix microarray analysis for indicated genes. The αGC- or OCH-stimulated liver NKT cells (purified as CD3+ NK1.1+ cells) as well as unstimulated NKT cells were analyzed at the indicated time points and the data represent to the relative values for glycolipid-treated samples when the value in NKT cells derived from untreated animals was defined as 1. (B) Real-time PCR analysis for the expression of CD40L mRNA. Data are presented as a fold induction of cytokine mRNAs after glycolipid treatment. The amount of mRNA in NKT cells derived from untreated animals was defined as 1. (C) Cell-surface expression of CD40L on αGC-stimulated (bold line) or OCH-stimulated (thin line) NKT cells. CD40L expression was analyzed in CD3/NK1.1 double-positive cell. Dotted line represents the histogram of control staining. B6 mice were injected intra-peritoneally with either αGC or OCH and liver mononuclear cells were isolated at the indicated time point. Cell-surface expression of CD40L was analyzed by flow cytometry (left) and plotted (right) as mean fluorescence intensity (MFI). Data are expressed as mean ± SD for duplicate samples. This figure represents one of two experiments with similar results. *$P < 0.05$.

OCH in a dose-dependent manner. Higher doses of IL-12 induced IFN-γ production even without OCH and the effect of OCH is concealed in this condition. Interestingly, IL-12 treatment inhibits IL-4 production by OCH-stimulated spleen cells in a dose-dependent manner, suggesting the reciprocal regulation of cytokine production by IL-12. Next we examined the effect of co-administration of sub-optimal dose of IL-12 together with OCH. As shown in Fig. 6(B), co-administration of OCH and IL-12 induced significantly higher production of IFN-γ compared with either treatment alone, although sub-optimal dose of IL-12 alone failed to induce IFN-γ production. In contrast, co-administration of IL-12 did not enhance the IL-4 production 2 h after OCH administration *in vivo.* As both NKT cells and NK cells are important sources of IFN-γ after glycolipid stimulation, we evaluated the frequency of IFN-γ-producing NKT and NK cells after co-administration of OCH with IL-12. As shown in Fig. 6(C), IL-12 augmented the proportions of IFN-γ-producing cells in both cell populations, but not in conventional T cells, when co-administered with OCH. These results demonstrated that the properties of OCH

for less effective IFN-γ production by NKT cells and NK cells could be compensated by co-administration of IL-12.

Modification of cytokine profiles by pathogen-associated molecular patterns after OCH treatment in vivo

As sub-optimal dose of IL-12 was able to rescue defective IFN-γ production by administration of OCH alone, availability of IL-12 might be a crucial determinant for OCH-induced production of IFN-γ. As DCs and phagocytes produce IL-12 in response to pathogens during infection, pathogen-associated molecular patterns (PAMPs) are possible important determinants for cytokine profiles after OCH stimulation *in vivo.* We applied CpG ODN (27), which skews the host's immune milieu in favor of T$_h$1 responses by enhancing the production of pro-inflammatory cytokines including IL-12 (28), for analyzing cytokine profile of OCH. As shown in Fig. 7(A), CpG ODN alone induced no cytokine production within 6 h after injection. Concomitant injection of CpG ODN with OCH induced strong IFN-γ production (7.5-fold induction with 10 µg per mouse of CpG ODN plus OCH and 14-fold induction with 100 µg per