PAXgene(BD Bioscience)を用いて RNA を精製、Geniom miRNA Biochip (febit; 866 miRNAs)を用いてmiRNA 発現を解析し、quantile normalization で正規化したデータセットである。

このデータに関して Welch's t-test と Bonferroni's correction で、MS 群と HC 群を比較し、両群間で有意な発現差異を認める miRNA を抽出した。すなわち p-Value < 0.05/866 = 0.0000577 を満たす 23 miRNAs を抽出した。さらに Cluster 3.0 と TreeView を用いて、階層クラスター解析を行い、Diana microT 3.0(diana.cslab.ece.ntua.gr/microT) を用いて、miTGscore > 20 を満たす信頼性の高い標的遺伝子を予測した。また DAVID6.7 gene ID conversion tool を用いて、標的遺伝子の Ensembl ID を Entrez Gene ID に変換、KeyMolnet(IMMD)に入力し、周辺検索法で分子ネットワーク(Pathway, Disease, Pathological Event との連関)を解析した。

(倫理面への配慮)

本研究では、公共データベース GEO に登録されているマイクロアレイ解析データを用いるため、倫理面の問題は考慮する必要がない。

C. 研究結果

MS 群と HC 群で有意な発現差異を認める 23 miRNAs を同定した(Table 1)。そのうち 9 miRNAs は既報(Keller et al. PLoS One 4: e7440, 2009)の most significantly deregulated miRNAs と一致した。MS で発現上昇は、miR-145, 186, 223, 1275, 92b＊, 664, 422a, 142-3p, 451, 942, 491-5p, 18＊, 151-3p, 22＊, 30e, 185＊である。MS で発現低下は、miR-20b, 216a, 107, 24, 330-3p, 103, 574-5p である。

階層クラスター解析では、miR-1275, 142-3p, 186, 22＊が MS 群と HC 群のクラスターを比較的よく識別する傾向を示した(Fig. 1)。

一つの miRNA による複数の標的遺伝子の同時発現制御機構に注目すると、信頼度が高い標的遺伝子が多数存在する miR-20b, 142-3p, 107, 103, 30e は、影響力が大きく機能的に重要である。miR-107(AGCAGCAUUGUACAGGGCUAUCA), miR-103(AGCAGCAUUGUACAGGGCUAUGA)は、1塩基のみ異なるが、標的遺伝子ネットワークは完全に一致していた。また配列が全く異なる miR-20b(CAAAGUGCUCAUAGUGCAGGUAG), miR-30e(UGUAAACAUCCUUGACUGGAAG)のネットワークがオーバラップしていた。MS で miR-20b は downregulation, miR-30e は upregulation を呈し、拮抗していた。KeyMolnet による解析では、Disease(疾患)は 2 型糖尿病や白血病との関連性が比較的高く、Pathological Event(病態イベント)は Cancer などとの関連性が比較的高い傾向を示した。MS で発現上昇していた miR-142-3p の 22 標的遺伝子の分子ネットワーク(Fig. 2)では、関連性が高いものとして、Transcriptional regulation by CLOCK/BMAL1(score = 245.613, p = 1.68E-51) および Prolactin signaling pathway(score = 118.861, p = 1.66E-36)が示唆された。

D. 考察

GSE17846 を解析し、MS 末梢血で有意な発現差異を認める 23 miRNAs を同定した。標的遺伝子が多数存在する miR-20b, 142-3p, 107, 103, 30e は、細胞機能に与える影響力が大きいと考えられる。

MS で発現低下していた miR-107, 103 は、標的遺伝子ネットワークが一致しており、ともに RSK signaling pattway の抑制に関与している可能性が示唆された (Table 1)。RSK は、ribosomal S6 kinase family であり、RSK1, RSK2, RSK3 から構成され、Ras-ERK-MAPK signaling cascade の下流に位置する Sr/Thr kinase である。増殖因子や TCR などの刺激で活性化され、様々な標的遺伝子をリン酸化することで、細胞の増殖や生存を制御する(Anjum and Blenis. Nat Rev Mol Cell boil 8: 747-758, 2008)。MS においては、Th17, Th1, Treg, B の細胞の増殖や生存の異常に関与している可能性がある。

MS で発現上昇し、MS 群と HC 群を良く識別した miR-143-3p の分子ネットワーク Prolactin (PRL) signaling pathway に関しては、MS では高 PRL 血症を認めるとの報告がある。PRL は乳腺発育と乳中分泌を刺激する下垂体前葉ホルモンで、Th1 による IFNG, IL-2 産生および Th2 による抗体産生を促進する(De Bellis et al. Pituitary 8: 25-30, 2005)。また PRL は oligodendrocyte progenitor cells (OPCs)の増殖を刺激し、髄鞘再生を促進する(Gregg. J Neurol Sci 285: 22-27, 2009)。MS における miR-143-3p を介する PRL signaling pathway の抑制は、PRL を介する neuroendocine system と immune system のリンクの異常と髄鞘再生不良に関与している可能性がある。

E. 結論

MS 末梢血で有意な発現差異を認める 23 miRNAs を同定した。これらのうちから、MS のバイオマーカーとなりうる miRNA を一つずつ検証する必要がある。

F. 健康危険情報

G. 研究発表

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H. 知的所有権の取得状況

1. 特許取得

該当なし

2. 実用新案登録

該当なし

3. その他

なし

MIRNA	MS vs HC Ratio	Regulation	p-Value	Targets Detected	Molecules of Network	Pathway			Disease			Pathological Event		
						Pathway	Score	p-Value	Disease	Score	p-Value	Event	Score	p-Value
hsa-miR-145	2.73	up	8.869E-10	3	9	NMDAR signaling pathway	17.883	4.14E-06	Type 2 Diabetes Mellitus	10	9.77E-04	Memory Consolidation	15.658	1.04E-05
hsa-miR-186	3.43	up	2.721E-09	5	22	Transcriptional regulation by HNF	44.381	4.37E-14	Hyperleucine-Isoleucinemia	28.638	2.41E-09	Cancer Cachexia	16.832	8.57E-06
hsa-miR-30b	0.49	down	5.683E-08	196	961	Transcriptional regulation by RB/E2F	961.124	2.36E-290	Chronic Myelogenous Leukemia	97.898	3.39E-30	Cancer	183.224	6.96E-56
hsa-miR-216a	0.63	down	1.02E-07	0	0									
hsa-miR-223	2.67	up	1.706E-07	1	12	Wnt signaling pathway	26.668	1.88E-08	Chronic Myelogenous Leukemia	19.8	1.10E-06	Mechanotransduction	28.893	1.09E-08
hsa-miR-1275	2.02	up	5.629E-07	0	0									
hsa-miR-92b*	2.31	up	6.381E-07	0	0									
hsa-miR-664	2.26	up	8.94E-07	0	0									
hsa-miR-421a	1.94	up	1.843E-06	0	0									
hsa-miR-142-3p	6.86	up	2.072E-06	22	169	Transcriptional regulation by CLOCK/BMAL1	168.67	1.68E-61	Marfan Syndrome	14.804	3.80E-05	Epithelial-Mesenchymal Transition	63.037	2.11E-19
hsa-miR-107	0.50	down	2.187E-06	45	188	RSK signaling pathway	246.613	1.16E-74	Adult T Cell Lymphoma/Leukemia	27.143	6.75E-09	Pol II Transcription	169.436	9.88E-52
hsa-miR-451	2.96	up	5.84E-06	0	0									
hsa-miR-942	2.78	up	6.65E-06	3	18	mTOR signaling pathway	76.875	7.22E-24	Hepatocellular Carcinoma	13.987	1.23E-04	Memory Consolidation	13.576	8.19E-05
hsa-miR-491-5p	1.60	up	1.729E-05	2	246	Lysophospholipid receptor signaling pathway	230.463	4.20E-70	Type 2 Diabetes Mellitus	17.421	5.70E-06	Itch	44.994	2.86E-14
hsa-miR-18a*	1.80	up	2.139E-05	0	0									
hsa-miR-24	0.55	down	3.367E-05	2	14	PAK signaling pathway	26.102	2.78E-08	Mantle-cell Lymphoma	17.466	5.52E-06	Endoplasmic Reticulum Stress	28.31	3.00E-09
hsa-miR-330-3p	0.55	down	3.446E-05	5	22	Wnt signaling pathway	49.564	1.20E-15	Chronic Myelogenous Leukemia	11.049	4.72E-04	Pituitary Function	34.904	3.19E-08
hsa-miR-103	0.60	down	3.521E-05	44	188	RSK signaling pathway	246.613	1.16E-74	Adult T Cell Lymphoma/Leukemia	27.143	6.75E-09	Pol II Transcription	169.436	9.88E-52
hsa-miR-151-3p	1.72	up	3.978E-05	0	0									
hsa-miR-22*	1.87	up	4.269E-05	0	0									
hsa-miR-30e	1.85	up	4.537E-05	455	1491	Transcriptional regulation by RB/E2F	780.29	1.29E-236	Chronic Myelogenous Leukemia	86.835	7.25E-17	Cancer	188.313	1.06E-57
hsa-miR-185*	6.42	up	4.747E-05	0	0									
hsa-miR-574-5p	0.44	down	5.822E-05	2	89	KLF family signaling pathway	390.043	3.86E-118	Mantle-cell Lymphoma	34.23	4.96E-11	Adipogenesis	61.614	1.81E-19

Table 1. MicroRNA and target networks deregulated in MS. GSE17846 は RRMS 患者(n =20)と healthy controls (HC; n =19)の末梢血全血の RNA に関して、Geniom miRNA Biochip (febit; 866 miRNAs)を用いて miRNA 発現を解析したデータセットである。このデータセットを用いて、Welch's t-test と Bonferroni's correction により MS 群と HC 群で有意な発現差異を認める 23 miRNAs を同定した。Diana microT 3.0 を用いて、miTGscore > 20 を満たす信頼性の高い標的遺伝子を予測し、標的遺伝子群に関して、KeyMolnet の周辺検索法で分子ネットワーク(Pathway, Disease, Pathological Event との連関)を解析した。

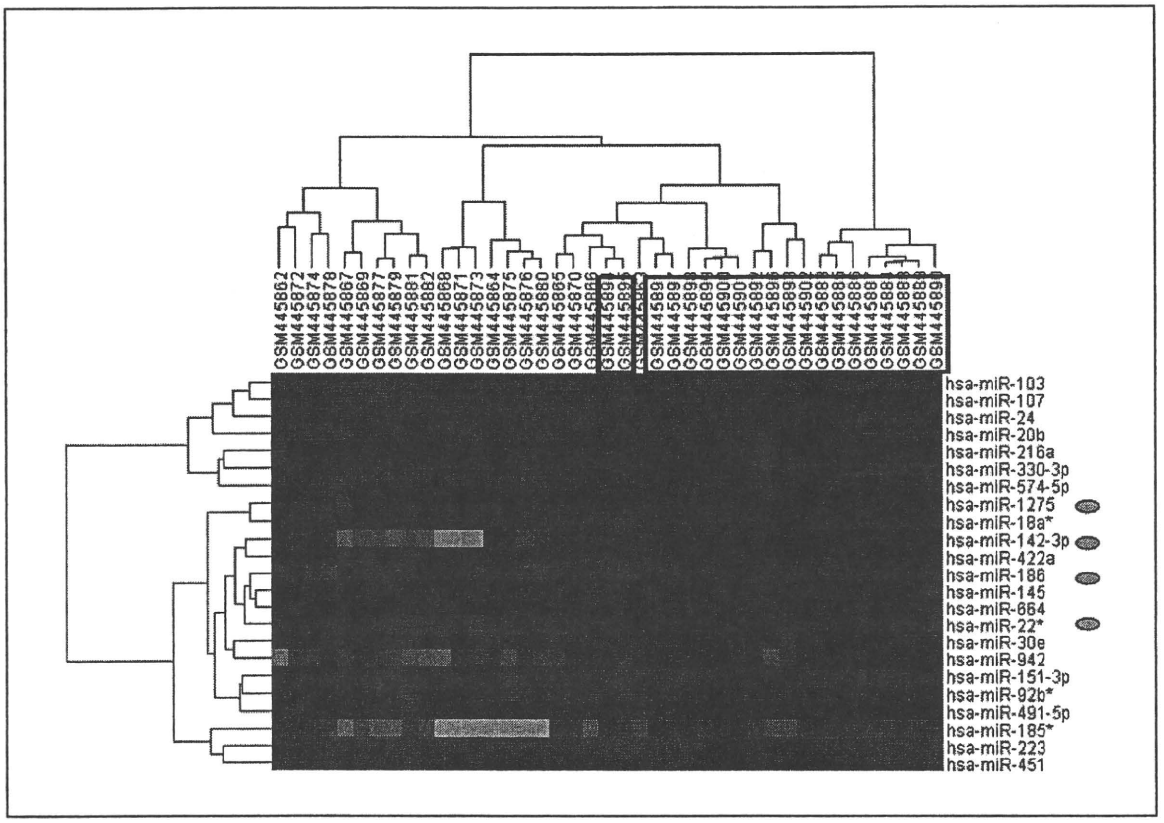

Fig. 1. Hierarchical clustering analysis of MS-specific miRNAs. MS 群と HC 群で有意な発現差異を認める 23 miRNAs に関して、Cluster 3.0 と TreeView を用いて、階層クラスター解析を行った。miR-1275, 142-3p, 186, 22＊が MS 群と HC 群のクラスターを比較的よく識別する傾向を示した(楕円)。

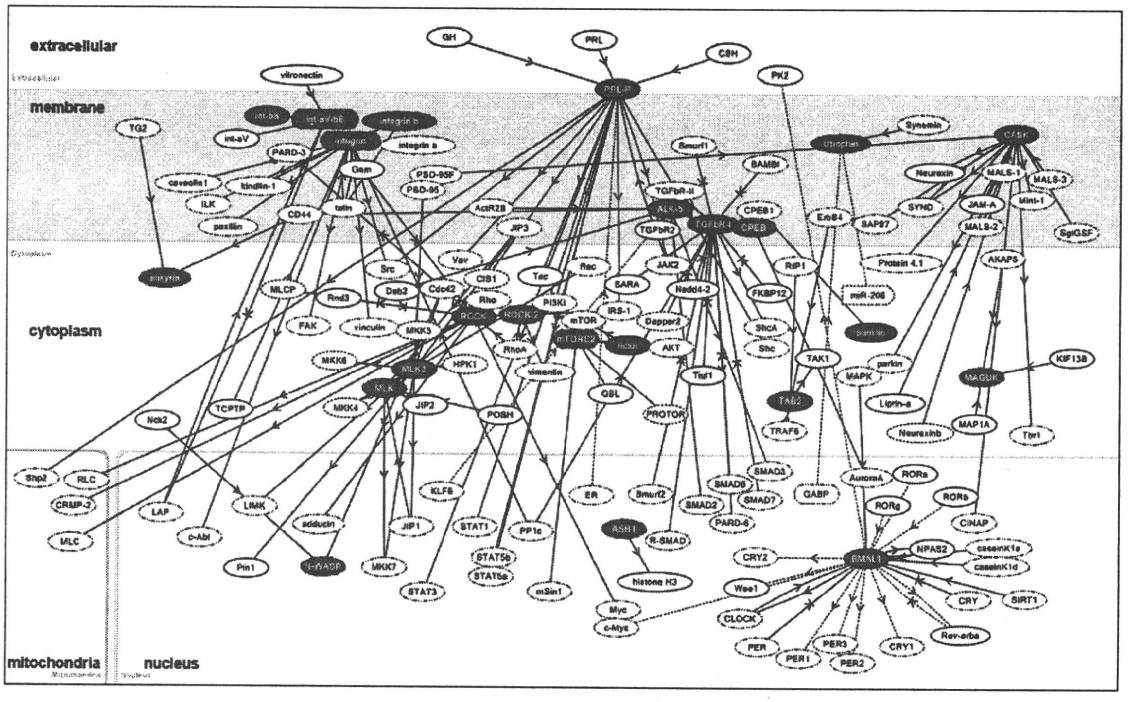

Fig. 2. miR-143p target network. MS で発現上昇していた miR-142-3p に関して Diana micro-T で予測した 22 標的遺伝子の分子ネットワークを KeyMolnet の周辺検索法で解析した。関連性が高い pathway として、Transcriptional regulation by CLOCK/BMAL1(score = 245.613, p = 1.68E-51)および Prolactin signaling pathway(score = 118.861, p = 1.66E-36)が示唆された。

Ⅲ.研究成果の刊行に関する一覧表

平成22年度研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名・出版地	頁	出版年
Sakuishi K, Miyake S, Yamamura T	Role of NK cells and Invariant NKT Cells in Multiple Sclerosis	Gramm U	Molecular Basis of Multiple Scoerosis. The Immune System Series "Results and Problems in Cell Differentiation"	Springer-Verlag, Heidelberg	127-147	2010

平成22年度研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表雑誌名	巻	頁	出版年
Chang, Y-J., H. Y. Kim, L. A. Albacker, H.-H. Lee, N. Baumgarth, S. Akira, P. Savage, S. Endo, T. Yamamura, J. Maaskant, N. Kitano, A. Singh, A. Bhatt, G. Besra, P. van den Elzen, B. Appelmelk, R. W. Franck, G. Chen, R. DeKruyff, M. Shimamura, P. Illarionov, and D. Umetsu	Influenza A infection in suckling mice expands a population of NKT cells that protects mice as adults from airway hyperreactivity	J. Clin. Invest.	121	57-69	2010
Satoh J	Bioinformatics approach to identifying molecular biomarkers and networks in multiple sclerosis.	Clinical and Experimental Neuroimmunology	1(3)	127-140	2010
Chihara N, Aranami T, Sato W, Miyazaki Y, Miyake S,Okamoto T, Ogawa M, Toda T, Yamamura T.	Interleukin 6 signaling promotes anti—aquaporin 4 autoantibody production from plasmablasts in neuromyelitis optica.	Proc Natl Acad Sci USA		in press	
荒浪利昌，山村　隆	炎症とT細胞サブセット．特集抗体療法	治療学	44(2)	11-13	2010
能登大介，山村　隆	免疫性神経疾患の免疫学	内科	105	756-761	2010
三宅幸子，山村　隆	NKT細胞と多発性硬化症	Mebio	27	94-101	2010
千原典夫、　山村　隆	神経疾患と炎症-多発性硬化症を中心に-	最新医学	65	2390-2395	2010
千原典夫、山村　隆	神経疾患と分子マーカー：多発性硬化症	Clinical Neuroscience	28	1396-1399	2010
冨田敦子、荒浪利昌、山村　隆	MSの免疫病態のトピックス	Brain Medical	22	25-30	2010
荒浪利昌、山村　隆	Th17細胞のケモカインレセプターの発現	Frontiers in Rheumatology & Clinical Immunology	4	28-32	2010
大木　伸司	核内受容体を標的としたTh17細胞制御と自己免疫疾患	生化学	82	745-750	2010

大木 伸司	多発性硬化症の病態解析から新規治療法の開発へ	ファルマシア	46	745-749	2010
吉村 元 大木伸司	Ustekinumabの有効性と疾患	Frontiers in Rheumatology & Clinical Immunology	4	57-60	2010

Ⅳ.研究成果の刊行物・別刷

Role of NK Cells and Invariant NKT Cells in Multiple Sclerosis

Kaori Sakuishi, Sachiko Miyake, and Takashi Yamamura

Abstract Natural killer (NK) cells and invariant natural killer T (*i*NKT) cells are two distinctive lymphocyte populations, each possessing its own unique features. Although NK cells are innate lymphocytes with cytotoxic property, they play an immunoregulatory role in the pathogenesis of autoimmune diseases. NKT cells are T cells expressing invariant TCR α-chains, which are known to bridge innate and adaptive arms of the immune system. Accumulating data now support active involvement of these cells in multiple sclerosis (MS). However, unlike professionally committed regulatory cells such as $Foxp3^+$ regulatory T cells, NK, and *i*NKT cells have dual potential of acting as either protective or pathogenic lymphocytes depending on the disease setting, adding complexity to the interpretation of data obtained from human and rodent studies. They are potential therapeutic targets in MS, and further in-depth understanding of these cells will lead to designing new strategies to overcome the disabling disease MS.

1 Introduction

Over the past years, a growing number of evidence has indicated that multiple sclerosis (MS) is as an autoimmune disease mediated by T cell immunity (Sospedra and Martin 2005). As described in detail in other chapters, pathogenesis of MS would actually involve autoreactive T cells that recognize the central nervous system (CNS) antigens. The target antigens include myelin basic protein (MBP) (Bielekova et al. 2000; Martin et al. 1991; Ota et al. 1990; Pette et al. 1990; Richert et al. 1989), myelin proteolipid protein (PLP) (Correale et al. 1995; Illes et al. 1999; Kondo et al. 1996; Ohashi et al. 1995; Pelfrey et al. 1993), and myelin oligodendrocyte glycoprotein (MOG) (Iglesias et al. 2001; Koehler et al. 2002; Mendel et al. 1995).

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Results Probl Cell Differ, DOI 10.1007/400_2009_11

Although the dominant role of $CD4^+$ T cells in MS has long been emphasized (Hafler 2004), more recent works indicate that $CD8^+$ T cells (Huseby et al. 2001; Skulina et al. 2004) and B cells also play a critical role in the disease development, and actually comprise a proportion of the CNS infiltrating cells. $CD8^+$ cells are reported to be predominant in the CNS lesions of MS, although compositions of cellular infiltrates vary greatly, depending on types and stages of this disease (Sospedra and Martin 2005). Now, the key question in MS lies in what disrupts the T cell and B cell immunological tolerance against the CNS antigens that are usually kept well secluded from the systemic immune system (Goodnow et al. 2005; Kyewski and Derbinski 2004; Walker and Abbas 2002). The relevance of this question is obvious because better understanding of the mechanism for the disruption of self-tolerance will lead to development of various new approaches to prevent the onset of MS and to control its further progression.

One of the distinctive and intriguing aspects of MS is that individual patients show various patterns in the longitudinal changes of its disease activity. While a large majority of the patients exhibit a relapsing and remitting course, some patients develop into or even start out as a progressive chronic illness (Sospedra and Martin 2005; Steinman 2001). Despite the vigorous efforts to control the activity of MS, currently available therapeutics to do not halt the progression of disease in a majority of cases, although some patients do not exhibit any sign of worsening for a long period of time even without treatment.

To clarify the regulation of autoimmune responses, much efforts have been dedicated to investigate the role of specialized adaptive regulatory T cells, including $CD4^+$ T cells expressing transcription factor Foxp3 (Miyara and Sakaguchi 2007), IL-10 producing T regulatory 1 (Tr1) cells (Roncarolo et al. 2006), and TGF-β producing Th3 cells (Awasthi et al. 2007; Baecher-Allan and Hafler 2006). However, recent publications provide evidence that cells of the innate immune system also have an unexpected potential to inhibit autoreactive $CD4^+$ T cells from mediating autoimmune disease and to protect tissues from collateral damage by T cells reactive to exogenous pathogens (Carrol and Prodeus 1998; Fearon and Locksley 1996; Medzhitov and Janeway 1997; Shi et al. 2001). Natural killer (NK) cells and invariant natural killer T (*i*NKT) cells, the main focus of this review, are also now recognized as innate cells with immunoregulatory potentials. Although they sense external ligands with different receptors (TCR for *i*NKT cells and NK receptor for NK cells), they behave like innate cells when they need to rapidly respond to stimuli. Therefore, it was believed previously that both cell types would primarily function within the innate arms of immunity. However, recent works have provided evidence that they would actively regulate T cell responses, thereby influencing the adaptive immune system (Bendelac et al. 1997; Carrol and Prodeus 1998; Fearon and Locksley 1996; Medzhitov and Janeway 1997; Shi et al. 2001; Shi and Van Kaer 2006).

In summary, NK cells and *i*NKT cells are now considered as multipotent cells that work at the border of innate and adaptive immunity, to prevent the induction, propagation, and activation of autoimmune T cells. Here, we review the latest advances in the research of the regulatory NK and *i*NKT lymphocytes with regard to the pathogenesis of MS and discuss the possibilities that they may serve as an effective target for MS therapy.

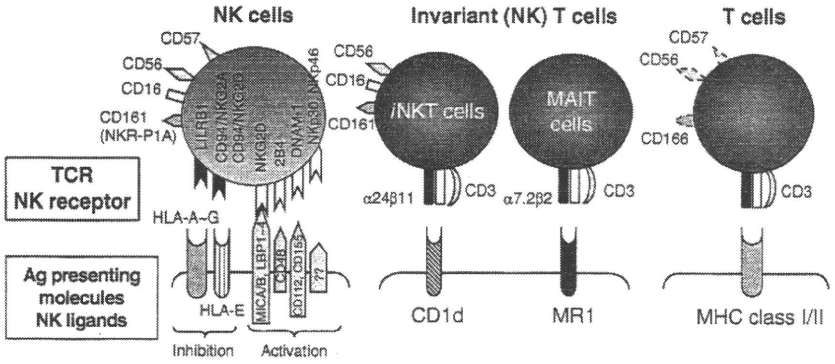
2 NK Cells and MS

2.1 General Properties of NK Cells

Natural killer cells are evolutionary primitive lymphocytes that lack antigen-specific receptors. They were originally identified as lymphoid cells capable of lysing tumor cell lines in the absence of prior stimulation *in vivo* or *in vitro*, which was the basis of their denomination (Trinchieri 1989). Constituting about 10% of the lymphocyte in human peripheral blood mononuclear cells (PBMC), NK cells possess cytotoxic properties, directed against virus-infected cells, thus considered as an important part of the innate immune system. Their cytotoxic reaction is determined by collective signaling of an array of inhibitory and stimulatory receptors expressed on their surface (Kirwan and Burshtyn 2007) (Fig. 1). Inhibitory receptors, commonly referred to as killer inhibitory Ig-like receptors (KIRs), interact with shared allelic determinants of classical and non classical MHC class I. Hence, NK cells are kept in an inactivated state through contact with self MHC class I molecule expressed on healthy cells. For example, CD94/NKG2A heterodimer expressed on NK cell surface recognize HLA-class Ib molecule, HLA-E (Borrego et al. 2006; Lopez-Botet et al. 1997). On the contrary, stimulatory receptors on NK cell surface bind to NK stimulatory receptor ligand up-regulated on other cells upon undergoing cellular stress. The main activating receptors constitutively found on all NK cells in peripheral blood are NKG2D, 2B4, and the two of the three natural cytotoxicity receptors (NCRs), NKp30, and NKp46. One example of NK stimulatory receptor ligand is the protein encoded by retinoic acid early inducible gene (RAE-I), which was isolated from tumor lines. RAE-1 is also expressed on virus-infected cells (Backstrom et al. 2007), and binds to the stimulatory receptor expressed on NK cells, NKG2D (Diefenbach et al. 2000; Smyth et al. 2005). As an overall effect, NK cells would lyse target cells that have lost or express low amounts of MHC class I molecules, including tumor cells or cells infected by viruses such as certain Herpes viruses or Adenoviruses.

Once activated, NK cells display cytotoxic functions which is mediated by direct cell-to-cell contact as well as secretion of cytokines and chemokines. The cell contact pathways include perforin/granzyme (Warren and Smyth 1999), Fas/Fas-ligand (Screpanti et al. 2005), and TRAIL/TRAIL ligand interaction (Takeda et al. 2001). They also produce inflammatory cytokines such as IFN-γ, TGF-β, and GM-CSF. Despite these cytotoxic actions against tumor cells and virus infected cells, it is now well conceived that some NK cells could act as modulator of adaptive immunity and have the potential to eliminate self-reactive T cells.

Although the diversity of NK cells remained to be ambiguous some time ago, recent works have greatly contributed to clarifying their heterogeneity in phenotypes and functions. The majority of human NK cells in PBMC belong to $CD56^{dim}CD16^{+}$ cytolytic NK subset. These cells express homing markers for inflamed peripheral sites and carry perforin to rapidly mediate cytotoxicity. $CD56^{bright}$ $CD16^{-}$ cells constitute a minor NK subset that lacks perforin but secrete large amounts of IFN-γ and



	Natural Killer cells	Invariant T cells		Conventional T cells
		*i*NKT cells	Vα7.2 *i*T cells	
TCR-Ag presenting molecules	None	α24β11- CD1d	α7.2β2/13- MR1	αβ- CD8:MHC class I CD4:MHC class II
NK marker	CD161 (NKR-P1) CD16 CD56 CD57 CD122	CD161 (NKR-P1) CD16 CD56	CD161 (NKR-P1)? CD16 ? CD56 ?	CD161, CD56, CD57 + in some subsets
NK receptor-ligands	Inhibition (KIR): CD94/NKG2A-HLA-E CD94/NKG2C-HLA-E LILRB1-HLA-A~G Activation: NKG2D-MICA/B ULBP1~4 NKp30- ??	CD94/NKG2A -HLA-E NKG2D-MICA/B ULBP1~4	??	Some cells pos. by induction
Memory phenotype		Majority CD69+	Majority CD69+	+ (Memory T cells)
Cytokine production	NK1: IFN-γ, TNF-α NK2: IL-5	DN: IFN-γ,TNF-α CD4: IL-4, IL-5, IL-13 (IL-17, IL-21)	? ? IFN-γ Th2 cytokine	CD8: IFN-γ CD4: Th1cell: IFN-γ Th2cell: IL-4, IL-5 Th17cell: IL-17
Perforine activation	+ (mainly NK1)	+ (mainly DN cells)	??	+ (mainly CD8 cells)
Frequency in PBMC	10 %	0.1 - 0.5 %	??	30-40 %

Fig. 1 Comparative features of human NK cells, invariant *i*NKT cells, and conventional T cells

TNF-α upon activation. They are superior to $CD56^{dim}$ cells in the regulatory functions that are mediated by these cytokines (Moretta et al. 2001). Moreover, they express surface markers such as CCR7 and CD62L that allow their homing to the lymph nodes, which results in the predominance of this NK cell subset in the secondary lymphoid organs.

Recent studies have shown that human NK cells are able to polarize *in vitro* into two functionally distinct subsets NK type 1 (NK1) or NK2 cells, analogous to T cell subsets Th1 or Th2. NK cells cultured in a condition favoring Th1 deviation (cultured with IL-12) would differentiate into NK1 cells producing IFN-γ and IL-10, whereas NK cells grown in a Th2 condition (cultured with IL-4) differentiate into NK2 cells producing IL-5 and IL-13 (Peritt et al. 1998). Although it was ambiguous whether the polarization actually occurs *in vivo*, an expansion of NK2 like cells producing IL-5 and IL-13 was observed in IFN-γ knockout mice (Hoshino et al. 1999), indicating that NK cells could functionally polarize into NK2-like cells in vivo.

Phenotypical analysis of NK cells in rodents has also identified a distinct population of NK cells that express CD11c, a prototypical dendritic cell (DC) marker. As the CD11c NK cells were shown to exhibit both NK and DC function, they are often referred to as "bitypic NK/DC cells" (Homann et al. 2002; Pillarisetty et al. 2005). CD11c molecule is known to be associated with integrin CD18 and form CD11c/CD18 complex. Although the precise function is not clear, CD11c is reportedly involved in binding of iC3b (Bilisland et al. 1994), adhesion to stimulated endothelium (Stacker and Springer 1991), and phagocytosis of apoptotic cells (Morelli et al. 2003). Bearing in mind that we have only very little knowledge of how these NK cell subsets are correlated to each other, we will next discuss on the recent progress which correlates the regulatory aspects of NK cells with the pathogenesis of MS.

2.2 NK Cell in MS

Despite the extensive studies in the past, there has been no simple uniform consensus regarding the role of NK cells in MS. Some of the earlier studies have found an inverse relationship between the number or the functional activity of circulating NK cells and the clinical or radiological activity of the patients with MS. NK cells isolated from MS patients were reported to be inefficient at cytotoxic killing and IFN-γ production (Benczur et al. 1980; Kastrukoff et al. 1998; Munschauer et al. 1995; Vranes et al. 1989). Furthermore, a longitudinal study showed that the functional activities of NK cells would decline during the relapse and then normalized during remission (Kastrukoff et al. 2003). On the contrary, several earlier studies failed to reveal any quantitative or qualitative difference between NK populations in MS patients versus controls (Hauser et al. 1981; Rauch et al. 1985; Rice et al. 1983; Santoli et al. 1981). The reason for these controversial findings remains to be unclear. However, it is of note that the criteria used to classify NK cells have been variable among the researchers and as a result the assays and protocols used to measure their functions and frequencies differ widely among the studies above mentioned. Moreover, because of difficulties in enrollment of patients, each of the studies might have examined the group of patients in different conditions. We also assume that they did not unify various confounding factors, some of which were not recognized when the study was conducted. Even duration of time between blood sampling and examination may affect the condition of NK cells (Takahashi et al. 2001).

In spite of the setbacks, the notion that NK cells have a significant role in reducing neuroinflammation and CNS injury stems from indirect evidences that were extracted from studies of an animal model experimental autoimmune encephalomyelitis (EAE) and from human clinical trials.

2.2.1 Protective Role of NK Cells in EAE

Monophasic EAE can be induced in C57BL/6 strain of mice (B6 mice) by immunizing the mice with an encephalitogenic myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}). When NK cells were depleted in vivo by antibody specific for NK1.1 molecule (CD161), mice developed an aggravated form of EAE in terms of onset and clinical severity (Zhang et al. 1997). Furthermore, NK cell depletion was found to increase proliferation and production of Th1 cytokines by memory $CD4^+$ T cells in the recall response to MOG. Similarly, NK cell depletion augmented the severity of EAE induced in β_2-microglobulin –/– mice. As the mice are lacking expression of CD1d molecule necessary for $NK1.1^+$ T cell development, it was assumed that NK cells would play a regulatory role in a manner independent of $NK1.1^+$ T cells. Furthermore, co-transfer of whole splenocytes, but not of NK cell-depleted splenocytes, ameliorated EAE that was induced by adoptive transfer of MOG-specific T cells into $Rag2^{-/-}$ hosts. Taken together, it was concluded that NK cells play a regulatory role in EAE. Involvement of NK cells was also demonstrated in Lewis rat EAE model which can be induced by sensitization to MBP (Matsumoto et al. 1998). When NK cells were depleted by antibody specific for either NKR-P1 (analogous to NK1.1) or asialo GM1, the rats developed an aggravated form of EAE, characterized by higher maximal clinical scores and increased mortality rates. Subsequently, Swanborg et al. have shown that rat bone marrow-derived NK cells would exhibit potent inhibitory effects on proliferation of auto-reactive T cells (Smeltz et al. 1999), further strengthening the postulate that NK cells play a regulatory role in the CNS autoimmunity.

More recently, Huang et al. have reported that mice deficient in CX3CR1 (the fractalkine receptor) develop a more severe form of EAE (Huang et al. 2006). Compared with their littermates, $CX3CR1^{-/-}$ mice immunized with MOG_{35-55} would exhibit a higher incidence of CNS hemorrhage, leading to a higher mortality rate. Moreover, the survived mice failed to recover neurological functions after they reached the peak of EAE. Although the $CX3CR1^{-/-}$ mice developed more serious manifestations of EAE, recall responses to MOG_{35-55} and generation of encephalogenic T cells in the peripheral lymphoid organs were not augmented in the mice. Notable differences were found in the CNS infiltrating cells. Namely, $NK1.1^+CD3^-$ cells were selectively depleted from mononuclear cells isolated from the spinal cord of the $CX3CR1^{-/-}$ mice, whereas they comprised 10–20% of the CNS infiltrates in wild-type mice and heterozygous $CX3CR1^{+/-}$ littermates. These findings led the authors to speculate that the exacerbated disease in $CX3CR1^{-/-}$ mice was due to a failure of regulatory NK cells to enter the target organ. In support of this, the majority of CNS-infiltrating NK cells in the littermate mice suffering from EAE expressed CX3CR1.

When NK cells were depleted in vivo by injecting anti-NK1.1 antibody, difference between $CX3CR1^{-/-}$ and the littermate $CX3CR1^{+/-}$ mice in the severity of EAE was no more evident. Of interest, soluble CX3CL1 was increased in the CNS of the EAE mice, and protein extracts from the CNS tissues showed a chemotactic activity for NK cells. It is of particular interest that a reduced number of circulating $CX3CR1^{+}$ NK cells has recently been reported in patients with MS (Infante-Duarte et al. 2005), which would prompt further investigation to examine a possible correlate between EAE and MS with regard to NK cell-mediated immunoregulation.

2.2.2 Ex Vivo Analysis Revealed an Alteration of NK cells in MS

Given putative roles of NK cells in MS, one may ask if there is a significant correlation of NK cell functions and the disease activity of MS. By analyzing surface phenotypes and cytokine secretion profile of peripheral blood NK cells, we demonstrated in 2001 that NK cells from MS patients during clinical remission are characterized by a higher frequency of $CD95^{+}$ cells as well as a higher expression level of IL-5, which represents a feature highly reminiscent of NK2 cells (Takahashi et al. 2001). The patients were selected from those who were not given any disease-modifying drugs, including corticosteroids. Remarkably, the NK2 cell-like feature, that is, a strong bias toward producing IL-5, was lost during the relapse of MS and regained after recovery. It was also found that NK2 cells induced *in vitro* from the peripheral blood of healthy subjects would inhibit the induction of Th1 cells, suggesting that the NK2 cells in vivo may also prohibit autoimmune effector T cells. Subsequently, we showed that when MS patients in remission are divided into two groups, according to the $CD95^{+}$ NK cell frequency, memory T cells reactive to MBP are increased in patients who possess a higher number of $CD95^{+}$ NK cells (Takahashi et al. 2004). Interestingly, NK cells from the "CD95 high patients" exhibited an ability to actively suppress the autoimmune T cells. These results allowed us to propose a model that CD95 low patients are enjoying very stable remission wherein an actual frequency of pathogenic autoimmune T cells is low, whereas CD95 high patients are in a more active state (which we call "smoldering state") wherein a higher number of autoreactive T cells are counter-regulated by NK cells (Fig. 2).

In a separate study, we found that CD11c expression on peripheral NK cells tends to correlate with temporal disease activity of MS (Aranami et al. 2006). Our study has revealed that surface CD11c expression on NK cells is significantly up-regulated in a proportion of patients with MS in remission, compared with healthy subjects or the rest of the patients. In the group of patients whose NK cells express higher levels of CD11c ("CD11c high patients"), IL-5 production from NK cells was significantly down-regulated and conversely, HLA-DR class II molecule was up-regulated. Accordingly, NK cells from "CD11c low patients" are NK2-biased, whereas those from "CD11c high patients" are not. NK cells from human PBMC would up-regulate expression of both CD11c and HLA-DR molecules after culture with IL-15 or a combination of IL-12 and IL-18 inflammatory cytokines commonly found in MS. Remarkably, the "CD11c high patients" tended to relapse significantly

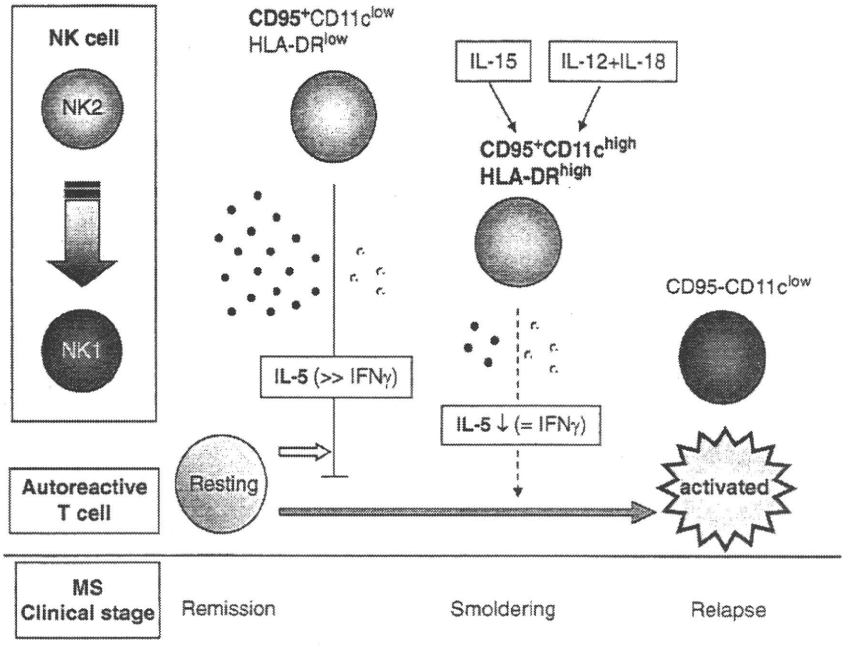


Fig. 2 Regulatory role of CD95+ NK 2 cell in MS remission

earlier than "CD11c low patients," indicating that "CD11c high patients" are clinically more active. We, therefore, propose that expression levels of CD11c on NK cells may serve as a good indicator of the disease activity (Fig. 2).

Another evidence for the role of NK cells in MS was obtained in the clinical trial of a new humanized monoclonal antibody against IL-2 receptor α-chain. In a recent phase II trial with the antibody (daclizumab), Bielekova et al. have noticed that an expansion of $CD56^{bright}$ immunoregulatory NK cells and their increased perforin expression would highly correlate with the reduction of the disease activity (Bielekova et al. 2006). In fact, contrast enhanced lesion on brain MRI was significantly suppressed along with an expansion of circulating $CD56^{bright}$ NK cells. NK cells isolated from patients being given daclizumab were found to exhibit cytotoxity towards autologous activated T cells, even without prestimulating NK cells with IL-2. These results raise a possibility that induced regulatory NK cells may at least partly mediate daclizumab effects on MS. In another study, an increase of $CD56^{bright}$ NK cells was demonstrated in the blood of newly diagnosed patients with relapsing-remitting MS who were started on interferon-β treatment a few months ago (Saraste et al. 2007). This work also supports a role for induced regulatory NK cells in patients who respond to immunomodulatory therapy. Taking the available data together, we assume that NK cells harbor functional subpopulations that play a protective role in CNS autoimmunity. Regulatory NK cells could be $CD56^{high}$, $CD95^+$, or $CX3CR1^+$, although mutual relationship of the populations still remains unclear. Further attempts to find a way to selectively activate regulatory NK cells are warranted, because it

will lead to developing a new treatment strategy for MS. It is known that NK cells show cytotoxic insults against CNS components in some *in vitro* conditions (Morse et al. 2001). To develop safe and effective drugs targeting NK cells, it is also important to know if regulatory NK cells could be selectively induced without augmenting cytotoxic NK cells that are potentially harmful for MS.

3 *i*NKT Cells in MS

3.1 What Is iNKT Cell?

3.1.1 General Properties of Invariant NKT (*i*NKT) Cells

Invariant NKT (*i*NKT) cells are a unique subset of lymphocytes that recognize a glycolipid antigen such as α-galactosylceramide (α-GC) (Kawano et al. 1997), that is bound to a monomorphic MHC class I-like molecule CD1d (Bendelac et al. 2007; Kronenberg 2005; Taniguchi et al. 2003). The term "NKT cells" was first introduced in mice to define a broader range of T cells that express the NK cell-associated marker NK1.1 (CD161) (Ballas and Rasmussen 1990; Fowlkes et al. 1987). The term "*i*NKT cells" defines a more limited population among $NK1.1^+$ T cells that express a single invariant α-chain (Vα14-Jα18 in mice and Vα24-Jα18 in humans) and respond to α-GC bound to CD1d (Dellabona et al. 1994; Exley et al. 1997; Koseki et al. 1991) (Fig. 1). The invariant α-chain is coupled with a noninvariant β-chain which selectively uses Vβ8.2, Vβ7, and Vβ2 gene segments in mice and Vβ11 (a molecule homologous to mice Vβ 8.2) in humans. It is currently known that mouse $NK1.1^+$ T cells (or NKT cells in the classic definition) are composed of *i*NKT cells, CD1d-restricted noninvariant T cells, conventional T cells that are not restricted by CD1d, and MAIT cells (see Sect. 4). On the other hand, there are a significant number of NK1.1-negative T cells that express the invariant Vα14-Jα18 TCR and react to α-GC/CD1d. In most of the current literatures, such T cells are also called *i*NKT cells.

*i*NKT cells constitutively express memory/activated T cell phenotype and are capable of robustly producing pro and antiinflammatory cytokines within hours after TCR engagement. The cytokine burst following *i*NKT cell activation then triggers a maturation process of downstream cells, such as NK cells, DCs, B cells, and T cells, which leads to subsequent alteration of a broad range of adaptive immune responses. Although *i*NKT cells utilize TCR for sensing a specific antigen, the behavior of the cells in response to external stimuli resembles that of innate lymphocytes (Mempel et al. 2002). Owing to the swift responsiveness to external stimuli, it is thought that *i*NKT cells play an important role in bridging innate and adaptive arms of immune response.

Another striking property of *i*NKT cells is to produce diverse combinations of cytokines, depending on how they are stimulated. Mouse *i*NKT cells can produce IFN-γ, IL-2–5, -13, -17, -21, GM-CSF, TNF-α, and osteopontin after an optimal engagement of TCR (Yamamura et al. 2007). In fact, they can produce a broad range