

Table 6. Enhancement of AFP-specific T cell response and treatment outcome

	Enhancement of AFP-specific T cell response	Recurrence, 3 months	Recurrence, 6 months
Patient 1	-	N	U
Patient 2	-	N	M
Patient 4	+	M	ND
Patient 5	-	N	M
Patient 6	+	N	U
Patient 9	-	N	M
Patient 10	-	N	N
Patient 13	-	N	N
Patient 14	-	N	N
Patient 16	-	N	M
Patient 19	-	N	U
Patient 24	+	U	ND
Patient 25	+	M	ND
Patient 26	+	N	N
Patient 30	+	N	N
Patient 31	+	N	N
Patient 33	-	N	N

Abbreviations: N, no recurrence; U, uninodular recurrence; M, multinodular recurrence; ND, not determined.

administration of DC in combination with tumor ablation.^{46,47} Furthermore, immunotherapies using DC have been performed in patients with HCC and their antitumor effects are reported.⁴⁸⁻⁵⁰ These results support our hypothesis and therefore, in the next step, we examined the immunological effects of DC infusion with TAE.

The comparison of frequency in patients who showed enhancement of AFP-specific immune responses revealed more frequency in patients with DC infusion than in those with TAE alone. On the other hand, there were no differences in the 2 groups in the comparison of frequency for patients who showed enhancement of CMV or TT-specific immune responses. These results suggest that DC infusion with TAE affects tumor-specific immune responses and that the effects are limited to the tumor area.

Some patients with TAE alone showed disappearance of AFP- or control antigen-specific T cells. Although the mechanism of this phenomenon is unknown, anticancer drugs used in TAE might suppress the immune responses, because most of the patients showed decreasing the number of lymphocytes after TAE. These results suggest that TAE alone might give a chance to enhance tumor-specific T-cell responses in only some patients. Further analysis using many more patients with TAE is necessary to make clear the differences in the patients with and without enhancement of T-cell responses. In contrast, disappearance of AFP- or control antigen-specific

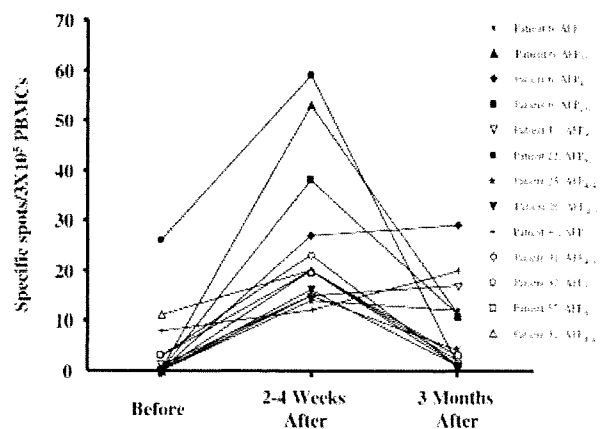


Figure 4. Kinetics of AFP-specific T-cell responses determined by IFN- γ ELISPOT assay before and after TAE. PBMCs were obtained before and 2-4 weeks and 3 months after TAE. Each graph indicates the kinetics of T cells specific for each antigen in each patient. Some patients received additional treatments as indicated in Tables 1 and 3 for a curative treatment after the measurement of T-cell responses at 2-4 weeks after TAE.

T cells was not observed in the patients with DC infusion, suggesting strong immunostimulating effect of this treatment.

In analysis of the association between the enhancement of AFP-specific T cells and clinical responses, no correlation could be shown, suggesting that enhancement of T-cell response associated with TAE or TAE with DC infusion may not have protective effect against HCC recurrence. To clarify the mechanism in more detail, we examined the kinetics of AFP-specific T-cell response. Increased frequency of AFP-specific T cells was transient and fell in 4 of 8 patients 3 months after treatment (Fig. 4). Similar to our results, Ayaru *et al.* also reported that the frequency of AFP-specific CD4⁺ T cells fell in all patients by 1-3 months after TAE.⁸ In addition, our results suggest that DC infusion with TAE is not effective to maintain the increased frequency of AFP-specific T cells.

Recent genome profiling studies of HCC show that HCC is a very heterogenous tumor.⁵¹ Furthermore, HCC has multicentric carcinogenesis and develops at different time points. These characters of HCC may also be another reason for no correlation between the enhancement of AFP-specific T cells and clinical responses. The identification of many more tumor antigens and their T-cell epitopes is necessary for more precise analysis of the relationship between anti-tumor immune response and clinical response, and for immunotherapy.

In the recent study, it is reported that CD8⁺ T-cell response to AFP is multispecific and AFP-specific IFN- γ -producing CD8⁺ T cells are directed against different epitopes spreading over the entire AFP sequence with no single

immuno-dominant CD8⁺ T-cell epitope.⁵² Therefore, there is a limitation to our study, because the number of immunogenic AFP-derived peptides applicable in this study is small. However, the results of the present study suggest that TAE with DC infusion enhances the tumor-specific immune responses. Although these modified immune responses may not be sufficient to prevent HCC recurrence because the

enhanced immune responses are transient and attenuate within 3 months, these results may contribute to the development of novel immunotherapeutic approach for HCC.

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References

- Curley SA, Izzo F, Ellis LM, Nicolas Vauthey J, Vallone P. Radiofrequency ablation of hepatocellular cancer in 110 patients with cirrhosis. *Ann Surg* 2000;232:381-91.
- Mazzaferro V, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, Montalto F, Ammatuna M, Morabito A, Gennari L. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N Engl J Med* 1996;334:693-9.
- Urabe T, Kaneko S, Matsushita E, Unoura M, Kobayashi K. Clinical pilot study of intrahepatic arterial chemotherapy with methotrexate, 5-fluorouracil, cisplatin and subcutaneous interferon-alpha-2b for patients with locally advanced hepatocellular carcinoma. *Oncology* 1998;55:39-47.
- Ishizaki Y, Kawasaki S. The evolution of liver transplantation for hepatocellular carcinoma (past, present, and future). *J Gastroenterol* 2008;43:18-26.
- Okuwaki Y, Nakazawa T, Shibuya A, Ono K, Hidaka H, Watanabe M, Kokubu S, Saigenji K. Intrahepatic distant recurrence after radiofrequency ablation for a single small hepatocellular carcinoma: risk factors and patterns. *J Gastroenterol* 2008;43:71-8.
- Abdel-Hady ES, Martin-Hirsch P, Duggan-Keen M, Stern PL, Moore JV, Corbitt G, Kitchener HC, Hampson IN. Immunological and viral factors associated with the response of vulval intraepithelial neoplasia to photodynamic therapy. *Cancer Res* 2001;61:192-6.
- Nakamoto Y, Mizukoshi E, Tsuji H, Sakai Y, Kitahara M, Arai K, Yamashita T, Yokoyama K, Mukaida N, Matsushima K, Matsui O, Kaneko S. Combined therapy of transcatheter hepatic arterial embolization with intratumoral dendritic cell infusion for hepatocellular carcinoma: clinical safety. *Clin Exp Immunol* 2007;147:296-305.
- Ayaru L, Pereira SP, Alisa A, Pathan AA, Williams R, Davidson B, Burroughs AK, Meyer T, Behboudi S. Unmasking of alpha-fetoprotein-specific CD4(+) T cell responses in hepatocellular carcinoma patients undergoing embolization. *J Immunol* 2007;178:1914-22.
- Zerbini A, Pilli M, Penna A, Pelosi G, Schianchi C, Molinari A, Schivazappa S, Zibera C, Fagnoni FF, Ferrari C, Missale G. Radiofrequency thermal ablation of hepatocellular carcinoma liver nodules can activate and enhance tumor-specific T-cell responses. *Cancer Res* 2006;66:1139-46.
- Mizukoshi E, Nakamoto Y, Tsuji H, Yamashita T, Kaneko S. Identification of alpha-fetoprotein-derived peptides recognized by cytotoxic T lymphocytes in HLA-A24+ patients with hepatocellular carcinoma. *Int J Cancer* 2006;118:1194-204.
- Gollnick SO, Evans SS, Baumann H, Owczarczak B, Maier P, Vaughan L, Wang WC, Unger E, Henderson BW. Role of cytokines in photodynamic therapy-induced local and systemic inflammation. *Br J Cancer* 2003;88:1772-9.
- Gollnick SO, Owczarczak B, Maier P. Photodynamic therapy and anti-tumor immunity. *Lasers Surg Med* 2006;38:509-15.
- Yamamoto N, Homma S, Sery TW, Donoso LA, Hooper JK. Photodynamic immunopotential: in vitro activation of macrophages by treatment of mouse peritoneal cells with haematoporphyrin derivative and light. *Eur J Cancer* 1991;27:467-71.
- den Brok MH, Suttmuller RP, van der Voort R, Binnink EJ, Figdor CG, Ruers TJ, Adema GJ. In situ tumor ablation creates an antigen source for the generation of antitumor immunity. *Cancer Res* 2004;64:4024-9.
- Kotera Y, Shimizu K, Mule JJ. Comparative analysis of necrotic and apoptotic tumor cells as a source of antigen(s) in dendritic cell-based immunization. *Cancer Res* 2001;61:8105-9.
- Sauter B, Albert ML, Francisco L, Larsson M, Somersan S, Bhardwaj N. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J Exp Med* 2000;191:423-34.
- Korbelik M, Sun J, Cecic I. Photodynamic therapy-induced cell surface expression and release of heat shock proteins: relevance for tumor response. *Cancer Res* 2005;65:1018-26.
- Takayasu K, Arai S, Ikai I, Omata M, Okita K, Ichida T, Matsuyama Y, Nakanuma Y, Kojiro M, Makuuchi M, Yamaoka Y. Prospective cohort study of transarterial chemoembolization for unresectable hepatocellular carcinoma in 8510 patients. *Gastroenterology* 2006;131:461-9.
- Matsui O, Kadota M, Yoshikawa J, Gabata T, Arai K, Demachi H, Miyayama S, Takashima T, Unoura M, Kogayashi K. Small hepatocellular carcinoma: treatment with subsegmental transcatheter arterial embolization. *Radiology* 1993;188:79-83.
- Yamada R, Kishi K, Sonomura T, Tsuda M, Nomura S, Satoh M. Transcatheter arterial embolization in unresectable hepatocellular carcinoma. *Cardiovasc Intervent Radiol* 1990;13:135-9.
- Pelletier G, Roche A, Ink O, Anciaux ML, Derhy S, Rougier P, Lenoir C, Attali P, Etienne JP. A randomized trial of hepatic arterial chemoembolization in patients with unresectable hepatocellular carcinoma. *J Hepatol* 1990;11:181-4.
- Groupe d'Etude et de Traitement du Carcinome Hépatocellulaire. A comparison of lipiodol chemoembolization and conservative treatment for unresectable hepatocellular carcinoma. *N Engl J Med* 1995;332:1256-61.
- Bruix J, Llovet JM, Castells A, Montana X, Bru C, Ayuso MC, Vilana R, Rodes J. Transarterial embolization versus symptomatic treatment in patients with advanced hepatocellular carcinoma: results of a randomized, controlled trial in a single institution. *Hepatology* 1998;27:1578-83.
- Llovet JM, Real MI, Montana X, Planas R, Coll S, Aponte J, Ayuso C, Sala M, Muchart J, Sola R, Rodes J, Bruix J. Arterial embolisation or chemoembolisation versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomised controlled trial. *Lancet* 2002;359:1734-9.
- Lo CM, Ngan H, Tso WK, Liu CL, Lam CM, Poon RT, Fan ST, Wong J. Randomized controlled trial of transarterial lipiodol chemoembolization for unresectable hepatocellular carcinoma. *Hepatology* 2002;35:1164-71.
- Hsu HC, Wei TC, Tsang YM, Wu MZ, Lin YH, Chuang SM. Histologic assessment of resected hepatocellular carcinoma after

- transcatheter hepatic arterial embolization. *Cancer* 1986;57:1184-91.
27. Kenji J, Hyodo I, Tanimizu M, Tanada M, Nishikawa Y, Hosokawa Y, Mandai K, Moriwaki S. Total necrosis of hepatocellular carcinoma with a combination therapy of arterial infusion of chemotherapeutic lipiodol and transcatheter arterial embolization: report of 14 cases. *Semin Oncol* 1997;24: S6-71-S6-80.
 28. Kobayashi N, Ishii M, Ueno Y, Kisara N, Chida N, Iwasaki T, Toyota T. Co-expression of Bcl-2 protein and vascular endothelial growth factor in hepatocellular carcinomas treated by chemoembolization. *Liver* 1999;19:25-31.
 29. Xiao EH, Li JQ, Huang JF. Effects of p53 on apoptosis and proliferation of hepatocellular carcinoma cells treated with transcatheter arterial chemoembolization. *World J Gastroenterol* 2004;10:190-4.
 30. Kanai M, Kohda H, Sekiya C, Namiki M. Effects on interleukin 1 alpha and beta production of peripheral blood mononuclear cells from patients with hepatocellular carcinoma after transcatheter arterial embolization. *Gastroenterol Jpn* 1990;25:662.
 31. Yamazaki H, Nishimoto N, Oi H, Matsushita M, Ogata A, Shima Y, Inoue T, Tang JT, Yoshizaki K, Kishimoto T, Inoue T. Serum interleukin 6 as a predictor of the therapeutic effect and adverse reactions after transcatheter arterial embolization. *Cytokine* 1995;7:191-5.
 32. Itoh Y, Okanoue T, Ohnishi N, Nishioji K, Sakamoto S, Nagao Y, Nakamura H, Kirishima T, Kashima K. Hepatic damage induced by transcatheter arterial chemoembolization elevates serum concentrations of macrophage-colony stimulating factor. *Liver* 1999;19:97-103.
 33. Araki T, Itai Y, Furui S, Tasaka A. Dynamic CT densitometry of hepatic tumors. *AJR Am J Roentgenol* 1980;135: 1037-43.
 34. Sobin LH, Wittekind C. TNM classification of malignant tumors, 6th edn. New York: Wiley-Liss, 2002. 81.
 35. Terayama N, Miyayama S, Tatsu H, Yamamoto T, Toya D, Tanaka N, Mitsui T, Miura S, Fujisawa M, Kifune K, Matsui O, Takashima T. Subsegmental transcatheter arterial embolization for hepatocellular carcinoma in the caudate lobe. *J Vasc Interv Radiol* 1998;9:501-8.
 36. Okamoto H, Shin J, Mion S, Koshimura S, Shimizu R. Studies on the anticancer and streptolysin S-forming abilities of hemolytic streptococci. *Jpn J Microbiol* 1967;11: 323-36.
 37. Nakahara S, Tsunoda T, Baba T, Asabe S, Tahara H. Dendritic cells stimulated with a bacterial product, OK-432, efficiently induce cytotoxic T lymphocytes specific to tumor rejection peptide. *Cancer Res* 2003; 63:4112-8.
 38. Japan LCSGo. Classification of primary liver cancer. English edn. 2. Tokyo: Kanehara, 1997.
 39. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994;19:1513-20.
 40. Mizukoshi E, Nakamoto Y, Marukawa Y, Arai K, Yamashita T, Tsuji H, Kuzushima K, Takiguchi M, Kaneko S. Cytotoxic T cell responses to human telomerase reverse transcriptase in patients with hepatocellular carcinoma. *Hepatology* 2006;43:1284-94.
 41. Mizukoshi E, Honda M, Arai K, Yamashita T, Nakamoto Y, Kaneko S. Expression of multidrug resistance-associated protein 3 and cytotoxic T cell responses in patients with hepatocellular carcinoma. *J Hepatol* 2008;49:946-54.
 42. Ikeda-Moore Y, Tomiyama H, Miwa K, Oka S, Iwamoto A, Kaneko Y, Takiguchi M. Identification and characterization of multiple HLA-A24-restricted HIV-1 CTL epitopes: strong epitopes are derived from V regions of HIV-1. *J Immunol* 1997;159: 6242-52.
 43. Kuzushima K, Hayashi N, Kimura H, Tsurumi T. Efficient identification of HLA-A*2402-restricted cytomegalovirus-specific CD8(+) T-cell epitopes by a computer algorithm and an enzyme-linked immunospot assay. *Blood* 2001;98:1872-81.
 44. Wissniewski TT, Hansler J, Neureiter D, Frieser M, Schaber S, Esslinger B, Voll R, Strobel D, Hahn EG, Schuppan D. Activation of tumor-specific T lymphocytes by radio-frequency ablation of the VX2 hepatoma in rabbits. *Cancer Res* 2003;63: 6496-500.
 45. Korbelik M, Krosli G, Krosli J, Dougherty GJ. The role of host lymphoid populations in the response of mouse EMT6 tumor to photodynamic therapy. *Cancer Res* 1996;56: 5647-52.
 46. Udagawa M, Kudo-Saito C, Hasegawa G, Yano K, Yamamoto A, Yaguchi M, Toda M, Azuma I, Iwai T, Kawakami Y. Enhancement of immunologic tumor regression by intratumoral administration of dendritic cells in combination with cryoablative tumor pretreatment and Bacillus Calmette-Guerin cell wall skeleton stimulation. *Clin Cancer Res* 2006;12: 7465-75.
 47. Machlenkin A, Goldberger O, Tirosh B, Paz A, Volovitz I, Bar-Haim E, Lee SH, Vadai E, Tzehoval E, Eisenbach L. Combined dendritic cell cryotherapy of tumor induces systemic antimetastatic immunity. *Clin Cancer Res* 2005;11: 4955-61.
 48. Ladhams A, Schmidt C, Sing G, Butterworth L, Fielding G, Tesar P, Strong R, Leggett B, Powell L, Maddern G, Ellem K, Cooksley G. Treatment of non-resectable hepatocellular carcinoma with autologous tumor-pulsed dendritic cells. *J Gastroenterol Hepatol* 2002;17: 889-96.
 49. Iwashita Y, Tahara K, Goto S, Sasaki A, Kai S, Seike M, Chen CL, Kawano K, Kitano S. A phase I study of autologous dendritic cell-based immunotherapy for patients with unresectable primary liver cancer. *Cancer Immunol Immunother* 2003; 52:155-61.
 50. Lee WC, Wang HC, Hung CF, Huang PF, Lia CR, Chen MF. Vaccination of advanced hepatocellular carcinoma patients with tumor lysate-pulsed dendritic cells: a clinical trial. *J Immunother* 2005;28: 496-504.
 51. Lee JS, Thorgeirsson SS. Genome-scale profiling of gene expression in hepatocellular carcinoma: classification, survival prediction, and identification of therapeutic targets. *Gastroenterology* 2004; 127:S51-5.
 52. Thimme R, Neagu M, Boettler T, Neumann-Haefelin C, Kersting N, Geissler M, Makowiec F, Obermaier R, Hopt UT, Blum HE, Spangenberg HC. Comprehensive analysis of the alpha-fetoprotein-specific CD8+ T cell responses in patients with hepatocellular carcinoma. *Hepatology* 2008;48:1821-33.

Crucial Contribution of Thymic Sirp α ⁺ Conventional Dendritic Cells to Central Tolerance against Blood-Borne Antigens in a CCR2-Dependent Manner

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Thymic dendritic cells (DCs) as well as thymic epithelial cells are presumed to be major sentinels in central tolerance by inducing the apoptosis of autoreactive T progenitor cells. The thymic DC population is composed of heterogeneous subsets including CD11c⁺B220⁺ plasmacytoid DCs, CD11c⁺B220⁻CD8 α ⁺ signal regulatory protein α (Sirp α)⁻ and CD11c⁺B220⁻CD8 α ⁻Sirp α ⁺ conventional DCs (cDCs). However, the distinctive role of each DC subset remains undefined. We show herein that Sirp α ⁺ cDCs, a minor subpopulation, was disseminated in the thymic cortical area with some of them uniquely localized inside perivascular regions and nearby small vessels in the thymus. The Sirp α ⁺ but not Sirp α ⁻ cDC subset can selectively capture blood-circulating Ags. Moreover, in CCR2-deficient mice, the thymic Sirp α ⁺ cDC subset, but not other thymic cell components, was moderately decreased especially in the perivascular regions. Concomitantly, these mice exhibited a modest impairment in intrathymic negative selection against blood-borne Ags, with the reduced capacity to uptake blood-borne Ags. Given their intrathymic cortical localization, CD11c⁺B220⁻CD8 α ⁻Sirp α ⁺ cDCs can have a unique role in the development of central tolerance against circulating peripheral Ags, at least partially in a CCR2-dependent manner. *The Journal of Immunology*, 2009, 183: 3053–3063.

The thymus is vital for development of T cells. T progenitor cells in the thymus are subjected to positive and negative selection, and survivors become self-MHC-restricted and self-tolerant mature naive T cells. Negative selection induces clonal deletion of potentially pathogenic autoreactive T cells and consequently decreases the risk of the development of autoimmune disorders (1). Thus, negative selection has a major role in central tolerance. Medullary thymic epithelial cells (mTECs)² are major inducers of negative selection. mTECs express the *autoimmune regulator* (*AIRE*) gene, which induces the ectopic expression of a milieu of peripheral tissue-specific Ags in the thymus resulting in the clonal deletion of autoreactive T progenitors with specificity for these Ags (2–4). Another type of thymic APCs, in particularly dendritic cells (DCs), have also been shown to contribute to negative selection (5–7). However, the detailed molecular and cellular mechanisms by which thymic DCs mediate negative selection remain largely unknown.

Thymic DCs are heterogeneous, similar to DCs in peripheral lymphoid organs such as lymph nodes and spleen. In humans and mice, thymic DCs are classified into two distinct subsets, CD11c⁺B220⁺ plasmacytoid DCs (pDCs) and CD11c⁺B220⁻

conventional DCs (cDCs). cDCs are further divided into CD11c⁺CD11b⁻CD8 α ⁺Sirp α ⁻ and CD11c⁺CD11b⁺CD8 α ⁻Sirp α ⁺ subsets (8, 9). CD8 α ⁺Sirp α ⁻ cDCs, the most abundant subset among these three thymic DC subsets, are clustered in the medulla (10, 11). These CD8 α ⁺Sirp α ⁻ cDCs also express *AIRE* and can present endogenous self-Ags. In addition, they can cross-present tissue-specific Ags derived from the mTECs for negative selection (12, 13). In contrast, the intrathymic location and functions of another minor cDC, CD11c⁺CD11b⁺CD8 α ⁻Sirp α ⁺, subset remain unclear, although this subset is presumed to migrate from the bloodstream (8). Proietto et al. (14) demonstrated that Sirp α ⁺ cDCs can induce thymocytes to efficiently differentiate into regulatory T cells in vitro. However, the roles of Sirp α ⁺ cDCs in central tolerance and regulatory T cell generation in vivo and the nature of the target autoantigens of central tolerance remain elusive.

Chemokines and their receptors have essential roles in controlling the homeostatic homing of immune cells including DCs and T cells (15–17). We examined the composition of thymic DC subsets in mice deficient in CCR1, CCR2, CCR5, or CX3CR1, the chemokine receptors which are expressed by DCs (18, 19). We observed that Sirp α ⁺ cDCs, but not Sirp α ⁻ cDCs or pDCs, were selectively decreased in the thymus of CCR2-deficient mice, but not in the other chemokine receptor gene-deficient mice. Interestingly, CCR2-deficient mice exhibited a modest impairment in intrathymic negative selection against i.v. injected Ags. Concomitantly, CCR2 deficiency allowed releasing more autoreactive T cells against serum Ags into periphery. These Sirp α ⁺ cDCs migrated from bone marrow to thymus by the way of the peripheral blood and showed a unique intrathymic localization confined to perivascular and cortical areas. Moreover, Sirp α ⁺ cDCs had a greater capacity to uptake blood-borne Ags than Sirp α ⁻ cDCs, along with their unique intrathymic localization. Thus, our present study suggests that thymic Sirp α ⁺ cDCs may function as a specialized APC for the development of central tolerance to blood-borne Ags.

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² Abbreviations used in this paper: mTEC, medullary thymic epithelial cell; DC, dendritic cell; Sirp α , signal regulatory protein α ; pDC, plasmacytoid DC; cDC, conventional DC; WT, wild type; Col IV, type IV collagen; FCM, flow cytometry; CMFDA, 5-chloromethylfluorescein diacetate; Cyt D, cytochalasin D; FSC, forward scatter; SSC, side scatter; DP, double positive.

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Materials and Methods

Mice

Specific pathogen-free 6- to 7-wk-old male BALB/c mice were purchased from Charles River Japan and designated as wild-type (WT) mice. $\text{CCR1}^{-/-}$ and $\text{CX3CR1}^{-/-}$ mice were provided by Dr. P. M. Murphy (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) (20, 21). $\text{CCR2}^{-/-}$ (22) and $\text{CCR5}^{-/-}$ mice (23) were provided by Dr. W. Kuziel (University of Texas San Antonio, San Antonio, TX) and Dr. Kouji Matsushima (University of Tokyo, Tokyo, Japan), respectively. All chemokine receptor-deficient mice were backcrossed to BALB/c mice for 8–10 generations. DO11.10 mice expressing a transgenic TCR that recognizes the OVA_{323–339} peptide in the context of I-A^d were maintained as heterozygotes. DO11.10 mice were backcrossed to $\text{CCR2}^{-/-}$ mice to generate DO11.10/ $\text{CCR2}^{-/-}$ mice. Genotyping for the CCR2 gene was done by direct PCR from whole blood samples using an Ampdirect Plus kit (Shimadzu) and the specific primers (sense, 5'-CACGAAGTATCCAAGAGCTTG-3' and antisense, 5'-CCCAAGTGAC TACACTTGTTA-3'). The mouse experiments were performed under specific pathogen-free conditions in accordance with the Guidelines for the Care and Use of Laboratory Animals of Kanazawa University.

Antibodies

Rat anti-mouse mAbs used were anti-CD3 ϵ (145-2C11; Miltenyi Biotec), anti-CD4 (RM4-5; BD Pharmingen), anti-CD8 (53-6.7; BD Pharmingen), anti-CD25 (PC61; BD Pharmingen), anti-CD45R/B220 (RA3-6B2; BD Pharmingen), anti-CD172a/ $\text{Sirp}\alpha$ (P84; BD Pharmingen), anti-DO11.10 clonotypic TCR (KJ1-26; BD Pharmingen), anti-F4/80 (A3-1; Serotec), and anti-Ly51 (6C3; BioLegend). Hamster anti-mouse CD11c (HL-3) and mouse anti-mouse I-A^d (AMS-32.1) mAbs were purchased from BD Pharmingen. Rabbit anti-mouse CCR2 mAb and anti-mouse type IV collagen (Col IV) polyclonal Ab were purchased from Epitomics and LSL, respectively. Goat anti-mouse MCP-2 polyclonal Ab was purchased from Santa Cruz Biotechnology. Isotype-matched control IgGs for each rat and hamster mAbs were purchased from BD Pharmingen. Mouse, rabbit, and goat IgG (Sigma-Aldrich) served as controls.

Cell preparation

Thymus was digested in 0.6 mg/ml collagenase type IV (Sigma-Aldrich) and 25 Kunitz units/ml DNase I (Sigma-Aldrich) in RPMI 1640 (Sigma-Aldrich) at 37°C for 20 min. The low-density cells were further isolated from the resultant single-cell suspensions using Histopaque-1077 reagent (Sigma-Aldrich). PBMCs were isolated from whole blood using Histopaque-1083 reagent (Sigma-Aldrich). Bone marrow cells were washed out with cold RPMI 1640 medium from the femoral and tibial bones.

Flow cytometry (FCM)

The low-density cells from thymus, PBMCs, and bone marrow cells were stained with various combinations of fluorescent dye-conjugated or non-conjugated specific Abs in PBS supplemented with 2 mM EDTA and 3% FBS. For nonconjugated Abs, fluorescent-conjugated secondary Abs were used. After washing in PBS, expression of cell surface molecular markers was analyzed using a FACSCalibur (BD Biosciences) with CellQuest Pro software (BD Biosciences).

Histology and fluorescent immunohistochemistry

Thymic tissues were frozen in OCT compound (Sakura) and 6- μm -thick cryostat sections were stained with H&E. For immunofluorescence analysis, 6- μm -thick cryostat sections were fixed with cold acetone for 3 min and incubated with Protein Block Reagent (DakoCytomation) to block nonspecific binding. Then fluorescent immunostaining was done by the standard method (for details, see the figure legends). After washing with 0.05% Tween 20-PBS, slides were mounted in fluorescent mounting medium (DakoCytomation). Immunofluorescence was detected in a setting that excluded the nonspecific signal of the isotype control using a fluorescence microscope (BX50; Olympus) or confocal laser-scanning microscope (LSM510; Zeiss). DP Controller software (Olympus) and Zen 2007 software (Zeiss) were used for image processing.

RT-PCR

Total RNAs were extracted from tissues using a RNeasy Mini Kit (Qiagen) and then reverse-transcribed using SuperScript III First-Strand Synthesis System (Invitrogen). PCR was done using the cDNAs, 2.5 mM dNTP mix (Takara), TaqDNA polymerase (Takara), and the specific primer sets for the GAPDH gene (sense, 5'-CAC TGA GCA TCT CCC TCA CA-3' and antisense, 5'-TGG GTG CAG CGA ACT TTA TT-3'), CD45 gene (sense,

5'-AAG ACA GAG TGC AAA GGA GAC-3' and antisense, 5'-TGT AGG TGT TTT CCC TGT GAC AAA GAC-3'), $\text{keratin } 8$ gene (sense, 5'-ACG GTG AAC CAG AGC CTG T-3' and antisense, 5'-CTC CAC TTG GTC TCC AGC AT-3'), MCP-1 gene (sense, 5'-CCC ACT CAC CTG CTG CTA CT-3' and antisense, 5'-TCT GGA CCC ATT CCT TCT TG-3'), MCP-2 gene (sense, 5'-CAG TCA CCT GCT GCT TTC AT-3' and antisense, 5'-ATA CCC TGC TTG GTC TGG AA-3'), and MCP-3 gene (sense, 5'-AAA CAA AAG ATC CCC AAG AGG-3' and antisense, 5'-CAC AGA CTT CCA TGC CCT TC-3') for 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s.

Effects of a peptide Ag on DO11.10 clonotypic thymocytes

DO11.10-transgenic mice with or without CCR2 gene deficiency were administered 200 μg of OVA_{323–339} peptide (ABGENT) in PBS through the tail vein. To induce thymocyte deletion independently of Ag presentation, mice were injected i.p. with 50 μg of anti-CD3 ϵ mAb (24). Two days after injection, thymocytes were collected and stained with the following combinations of Abs: anti-CD4, anti-CD8, and anti-DO11.10 or anti-CD4, anti-CD25, and anti-DO11.10 Abs. To detect apoptotic cells, thymocytes were stained using an Annexin V-FITC Apoptosis Detection Kit (Merck). After being stained, the cells were analyzed by FCM.

Trafficking of bone marrow-derived immature DCs injected into bone marrow

Bone marrow cells were cultured in RPMI 1640 medium supplemented with 10% FBS and mouse GM-CSF (R&D Systems) at a concentration of 20 ng/ml. An equal volume of culture medium of the same content was added at 4 days, and one-half of the medium was replaced with fresh culture medium at 7 days after the plating. Most bone marrow cells were differentiated into immature DCs as judged by morphological appearances at 10 days after the initiation of the culture. The resultant immature DCs were stained with 1 μM 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen) dye and 1 million cells were injected into the tibial bone marrow cavity of each mouse. After the injection, low-density cells were obtained from thymus, lymph nodes, or PBMCs to determine the presence of CMFDA-stained DCs by using FCM.

Localization of the i.v. injected Ags

Alexa Fluor 488-conjugated OVA protein (OVA₄₈₈), Alexa Fluor 647-conjugated OVA protein (OVA₆₄₇) (Invitrogen), or mouse serum IgG (Sigma-Aldrich), which was conjugated with Alexa Fluor 647, using an Alexa Fluor 647 protein labeling kit (Invitrogen), was injected into the tail vein of mice. Thymic low-density cells and PBMCs were isolated at the indicated time points after OVA protein injection and were stained with anti-CD11c and anti- $\text{Sirp}\alpha$ Abs. Then the cells were analyzed by FCM. For the localization of the Ag uptake, cryostat sections of frozen thymic tissues were obtained from mice injected with OVA protein and were stained with anti- $\text{Sirp}\alpha$, anti-CD11c, anti-I-A^d, anti-Ly51, or anti-Col IV Abs and were then observed by fluorescence microscope.

In vitro endocytosis assay

Low-density cells were isolated from the thymus and were incubated with 10 $\mu\text{g}/\text{ml}$ OVA₆₄₇ in RPMI 1640 at 37°C for 20 min. As a negative control, incubation was conducted on ice. Endocytosis by each thymic DC subset was analyzed by FCM after being stained with anti-CD11c and anti- $\text{Sirp}\alpha$ Abs. In some experiments, low-density cells were preincubated with 10 μM cytochalasin D (Cyt D; Sigma-Aldrich), an actin inhibitor (25), 100 mM ammonium chloride (NH_4Cl) (Wako), an inhibitor of the clathrin-dependent pathway (26), or 0.5 mg/ml mannan (Sigma-Aldrich) at 37°C for 15 min before incubation with OVA₆₄₇ at 37°C for 20 min in the presence of fresh inhibitors.

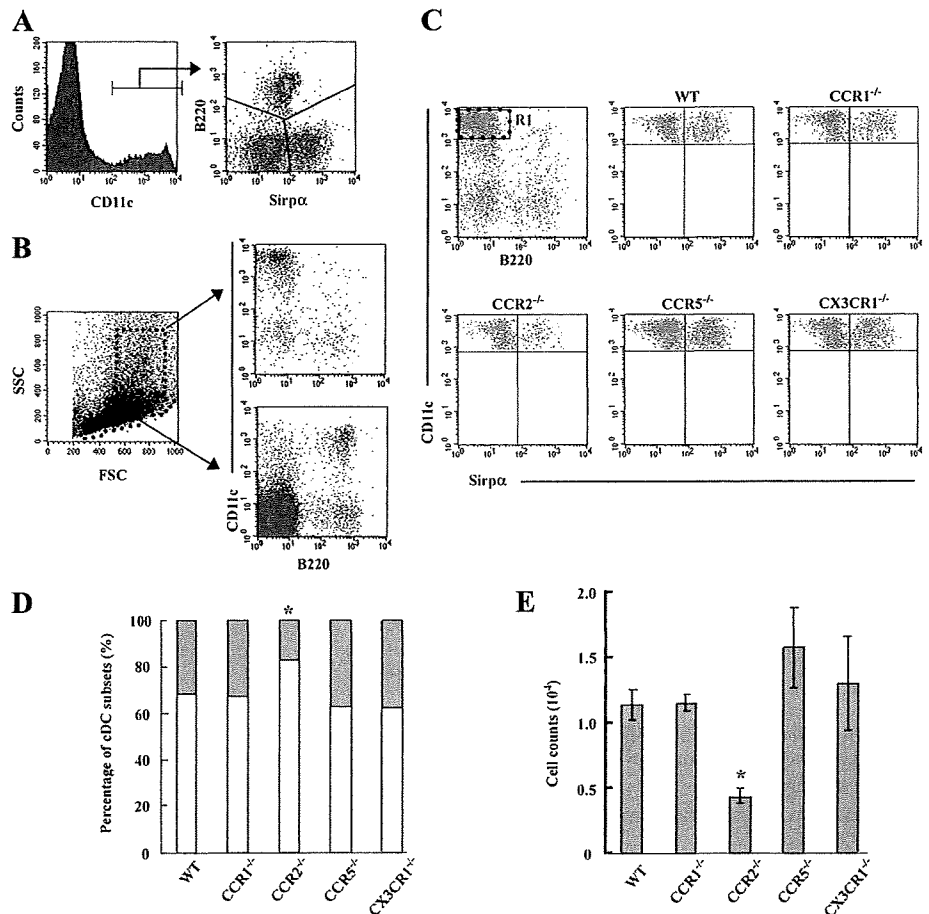
Adoptive transfer of bone marrow cells

Bone marrow cells were obtained from WT or $\text{CCR2}^{-/-}$ mice and were stained with 2 μM CMFDA dye. Twenty million cells were injected into the tail vein of $\text{CCR2}^{-/-}$ mice. OVA₆₄₇ was injected into the tail vein at 2 days after injection. Thymic low-density cells were isolated at 1 h after OVA protein injection, and the presence of donor-derived $\text{Sirp}\alpha^+$ cDCs and their capability of Ag uptake were analyzed by FCM.

In vivo cell proliferation assay

Spleen mononuclear cells were isolated from WT or $\text{CCR2}^{-/-}$ mice and were labeled with 25 μM CFSE using a CellTrace CFSE Cell Proliferation Kit (Invitrogen). Ten million prelabeled cells were injected into the tail vein of WT mice. One day after injection, mice were immunized with total

FIGURE 1. Effects of chemokine receptor deficiency on mouse thymic DC subsets. **A**, Low-density cells were isolated from WT mouse thymus and were stained with PE-conjugated anti-CD11c, allophycocyanin-conjugated anti-B220, and nonconjugated anti-Sirp α mAbs, followed by staining with FITC-conjugated mouse anti-rat IgG1. The CD11c⁺ DC populations were gated to analyze the expression of Sirp α and B220. **B**, Thymic low-density cells were divided into two groups based on their FSC and SSC patterns, which are indicated by elliptic and square gates. Then DC subsets in each region were analyzed. **C**, Low-density cells were isolated from WT, CCR1^{-/-}, CCR2^{-/-}, CCR5^{-/-}, and CX3CR1^{-/-} mice. The Sirp α ⁻ and Sirp α ⁺ subsets in FSC^{high} SSC^{high}CD11c^{high}B220⁻ cDC populations gated with region 1 (R1) were compared among these mice. **D**, The ratio of two DC subsets (blank portion, Sirp α ⁻ subset; gray portion, Sirp α ⁺ subset) present in thymic cDC population was determined. Data represent the mean of three independent experiments. **E**, The numbers of Sirp α ⁺ DCs in the thymus. Data represent mean \pm SD from three independent experiments. *, $p < 0.01$.



mouse serum protein emulsified in CFA. PBS in CFA was immunized as a control. Two days after immunization, lymphocytes were harvested from draining and nondraining lymph nodes and stained with anti-CD4 mAb. The percentage of CFSE-diluted divided cells was analyzed by FCM.

Statistical analysis

Data are represented as mean \pm SD. Statistical significance was determined by one-way ANOVA followed by the Tukey-Kramer test. A value of $p < 0.05$ was considered statistically significant.

Results

Selective reduction of thymic Sirp α ⁺ cDCs in CCR2^{-/-} mice

Consistent with a previous report (8), three distinct populations of thymic CD11c⁺ DCs have been identified: B220⁺ pDC, B220⁻ Sirp α ⁻ cDC, and B220⁻ Sirp α ⁺ cDC subsets (Fig. 1A). cDC and pDC subsets were present mainly in the forward scatter (FSC^{high}), side scatter SSC^{high}, and SSC^{low} areas upon FCM, respectively (Fig. 1B). The pivotal role of chemokines in the trafficking of DCs prompted us to examine thymic DC subsets in mice deficient in chemokine receptor genes. Sirp α ⁺ DCs were markedly decreased in CCR2^{-/-} mice, compared with WT mice, both in the relative (Fig. 1, C and D) and absolute number (Fig. 1E), whereas Sirp α ⁻ DC (Fig. 1C) and B220⁺ pDC numbers (data not shown) were not changed in CCR2^{-/-} mice. In contrast, no significant changes were observed on thymic cDC and pDC subsets in mice deficient in other chemokine receptors including CCR1, CCR5, and CX3CR1. Moreover, we did not observe any differences in thymic B220⁺ B cell and F4/80⁺ macrophage numbers between WT and CCR2^{-/-} mice (data not shown). Microscopic studies of the thymus failed to reveal any morphological differences between WT

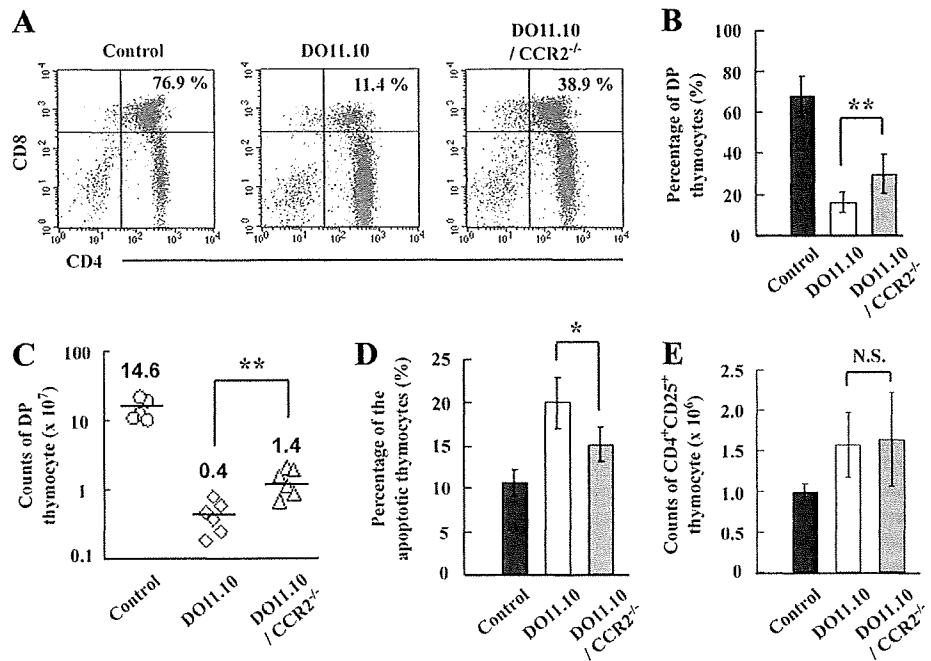
and CCR2^{-/-} mice in terms of the total cellularity, the distribution of thymocytes in each developmental stage, and the localization of Ly51⁺ cortical thymic epithelial cells and I-A^d high mTEC (supplemental Fig. S1³). Thus, CCR2^{-/-} mice exhibit a selective decrease in the Sirp α ⁺ DC subset in thymus.

Attenuation of OVA₃₂₃₋₃₃₉ peptide-induced clonal deletion by CCR2 gene ablation

Sirp α ⁺ DCs are presumed to have the capacity to carry peripheral tissue Ags into the thymus (14). We next investigated the roles of Sirp α ⁺ DCs in thymus on taking in an i.v. administered Ag. PBS injection did not cause any changes in each developmental stage of thymocytes in DO11.10 and DO11.10/CCR2^{-/-} mice (data not shown). On the contrary, i.v. administration of OVA₃₂₃₋₃₃₉ peptide markedly reduced the proportion and absolute number of clonotypic CD4/CD8 double-positive (DP) thymocytes in DO11.10 mice. CCR2 gene ablation modestly attenuated this reduction (Fig. 2, A–C). OVA peptide injection consistently increased the proportion of annexin V⁺ apoptotic cells in DO11.10 mouse thymus compared with that in DO11.10/CCR2^{-/-} mice (Fig. 2D). In contrast, OVA peptide induced a modest increase in the number of DO11.10⁺ CD25⁺ CD4⁺ regulatory T cell phenotype to similar extents in both DO11.10 and DO11.10/CCR2^{-/-} thymus (Fig. 2E). Thus, decreased thymic Sirp α ⁺ DCs in CCR2^{-/-} mice may be associated with a moderately impaired thymic negative selection. Moreover, following i.p. injection with anti-CD3 Ab (24), thymocytes were deleted to similar extents in DO11.10 and DO11.10/CCR2^{-/-} mice

³ The online version of this article contains supplemental material.

FIGURE 2. Induction of clonal deletion of DO11.10 clonotypic thymocytes. To induce the clonal deletion, 200 μ g of OVA_{323–339} peptide in PBS was injected into the tail vein of DO11.10-transgenic or DO11.10/CCR2^{-/-} mice. PBS was injected as a control. DO11.10-transgenic TCR-expressing thymocytes were identified as KJ1-26-positive cells. **A**, Each developmental stage of thymocytes after OVA_{323–339} peptide injection. Percentage of DP stage is shown in each panel. **B**, Percentage of DP stage of development; **C**, the number of DP thymocytes; **D**, percentage of the apoptotic thymocytes; and **E**, the number of CD4⁺CD25⁻ thymocytes were determined on DO11.10 and DO11.10/CCR2^{-/-} mice. Representative results from at least four independent experiments are shown in **A** while the mean \pm SD was calculated on at least four independent experiments and are shown in **B–E**. *, $p < 0.05$ and **, $p < 0.01$. N.S., No significant difference.



(supplemental Fig. S2), indicating the absence of intrinsic defects of thymocytes in the absence of CCR2. These results collectively suggest that thymic $Sirp\alpha^+$ DCs can contribute to intrathymic negative selection of a bloodstream-derived Ag without inducing regulatory T cells.

Thymic $Sirp\alpha^+$ DCs can efficiently capture peripheral Ag from bloodstream

To elucidate the functions of thymic $Sirp\alpha^+$ DCs more in detail, we determined their intrathymic localization. In thymi of WT mice, $Sirp\alpha$ was mainly detected on CD11c⁺ DCs scattered in the thymic cortex (Fig. 3, A and B), but not on CD11c⁻ DCs clustered in medulla, the predominating site of thymic CD8 α^+ $Sirp\alpha^-$ DCs. Moreover, most $Sirp\alpha^+$ DCs were localized in close proximity to small vessels with single Col IV⁺ basement membrane or inside perivascular regions (PVRs) separated by two Col IV⁻ basement membranes in the cortex (Fig. 3C). The thymic DC population includes APCs crucially involved in the central tolerance system involving bloodstream C5 Ag (27). Furthermore, $Sirp\alpha^+$ DCs are selectively localized in PVRs or in close proximity to small vessels, both essential components of the blood-thymus barrier (28). Hence, we hypothesized that this DC subset might be involved in Ag uptake from the bloodstream. To address this possibility, we treated WT mice i.v. with OVA₆₄₇ and examined its uptake by thymic DCs. Intrathymic $Sirp\alpha^+$ DCs, but not $Sirp\alpha^-$ DCs, took up OVA protein in a dose-dependent manner (Fig. 4A), maintaining a stable level from 1 to 4 h after the injection and decreasing thereafter (Fig. 4B). Recently, it was reported that bloodstream DCs could efficiently capture and transport particulate bacteria into the spleen when particulate bacteria were i.v. injected (29). Indeed, bloodstream CD11c⁺ cells rapidly disappeared from the peripheral blood after capturing OVA protein (Fig. 4C). By contrast, the uptake by intrathymic $Sirp\alpha^+$ DCs reached a peak level at 15 min, decreasing to the stable level thereafter. Thus, there may be a remote possibility that circulating DCs migrated into the thymus after capturing OVA protein inside the bloodstream. Furthermore, in addition to an exogenous protein, intrathymic $Sirp\alpha^+$ DCs also captured an endogenous serum protein, mouse IgG, which was

conjugated with Alexa Fluor 647, when it was administered i.v. (supplemental Fig. S3). Thus, $Sirp\alpha^+$ DCs can effectively capture peripheral Ags from the bloodstream across the blood-thymus barrier. This notion was further supported by the observation that $Sirp\alpha^+$ DCs engulfed OVA protein with a higher efficiency than $Sirp\alpha^-$ DCs when cultured in vitro with OVA₆₄₇ (Fig. 4, D and E). Mannan from *Saccharomyces cerevisiae*, but not NH₄Cl or Cyt D from *Zygosporium mansonii*, markedly inhibited endocytosis of OVA protein by $Sirp\alpha^-$ DCs (Fig. 4F, upper panel). On the contrary, uptake of OVA protein by $Sirp\alpha^+$ DCs was markedly attenuated by NH₄Cl and Cyt D, but not mannan (Fig. 4F, lower panel). These observations suggest that thymic $Sirp\alpha^+$ DCs can endocytose soluble Ags more efficiently than $Sirp\alpha^-$ DCs, in a clathrin-dependent, but not mannose receptor-dependent manner.

Thymic $Sirp\alpha^+$ DCs capture peripheral Ag inside PVRs or nearby small vessels, and then migrate into the cortical parenchyma

We examined sequentially intrathymic localization of OVA-derived signals after i.v. injection of OVA₄₈₈. By 0.5 h, OVA₄₈₈-derived signals were detected in $Sirp\alpha^+$ cells (Fig. 5A), CD11c⁺ DCs (Fig. 5B) and inside PVRs or in close proximity to small vessels (Fig. 5C). Although some signals remained nearby in small vessels, signals inside PVRs were obviously decreased at 6 h (Fig. 5D), as judged by the Col IV immunostaining pattern. At 18 h after the injection, OVA₄₈₈-derived signals were mainly scattered in the Ly51⁺ cortical area but not in the I-A^d high medullary area (Fig. 5E). Because OVA₄₈₈-derived signals were constantly detected in $Sirp\alpha^+$ DCs at every time point (data not shown), these observations suggest that $Sirp\alpha^+$ DCs initially capture bloodstream OVA protein inside PVRs or in nearby small vessels and then migrate into the cortical parenchyma. To examine the process of migration more in detail, OVA₆₄₇ (blue) and OVA₄₈₈ (green) were i.v. injected sequentially with an interval of either 6 or 18 h as shown in Fig. 5F. When OVA₄₈₈ was injected 6 h after OVA₆₄₇, double-positive CD11c^{high} DCs were evidently detected (8.1%), while single-positive cells were sparse (Fig. 5F, left upper panel). Even at 18 h after the injection, double-positive CD11c^{high} DCs were still

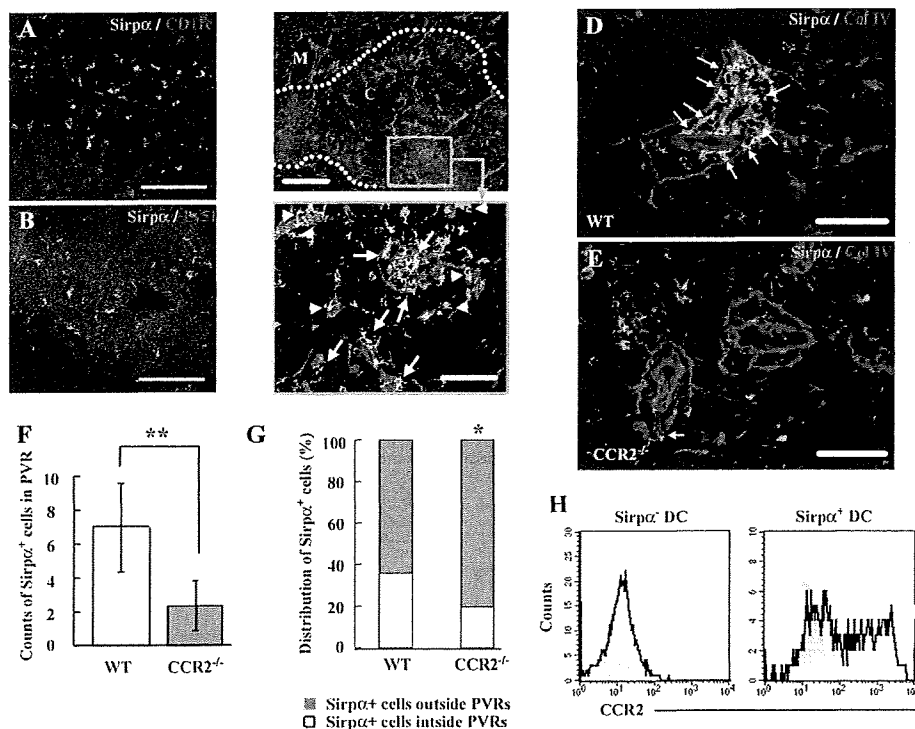


FIGURE 3. Localization of thymic Sirp α ⁺ DCs. Double-color fluorescence immunostaining for Sirp α (green) and CD11c (red; A), Ly51 (red; B), or Col IV (red; D and E). C, Triple-color fluorescent immunostaining for Sirp α (green), Ly51 (red), and Col IV (blue). Low magnification image for Ly51 and Col IV is shown in the upper panel. A green square in the upper panel is observed at a higher magnification for Col IV and Sirp α expression and is shown in the lower panel. Dashed lines indicate the boundary between cortex (C) and medulla (M). Arrowheads in C and arrows in C–E indicate Sirp α ⁺ cells interacting with small vessels and inside the PVRs, respectively. A–D, WT thymus. E, CCR2^{-/-} thymus. Representative results from at least two independent experiments are shown here. Scale bars: A and B, 100 μ m; upper panel of C, 200 μ m; lower panel of C–E, 50 μ m. F and G, At least five photographs in the central regions of the PVRs were taken at \times 200 magnification in each tissue sample. The numbers of Sirp α ⁺ cells inside the PVRs were determined and data represent mean \pm SD of three independent experiments. **, $p < 0.01$. F, The proportion of Sirp α ⁺ cells inside the PVRs to outside was calculated and data represent the mean of three independent experiments. *, $p < 0.05$ (G). H, CCR2 expression on CD11c^{high}Sirp α ⁺ or Sirp α ⁺ cDCs. Gray-filled and black-open histograms indicate the results from isotype control and specific mAb for CCR2, respectively. Representative results from three independent experiments are shown here.

present (3.2%) with substantial numbers of OVA₄₈₈-derived signal single-positive (3.9%) or OVA₆₄₇-derived signal single-positive cells (2.3%; Fig. 5F, left lower panel). Thus, CD11c^{high} DCs with Sirp α expression can persistently be in close interaction with the bloodstream while they are migrating into cortical parenchyma (Fig. 5G).

Depressed migration of Sirp α ⁺ DCs and their aberrant intrathymic localization in CCR2^{-/-} mice

It is possible that a decreased intrathymic Sirp α ⁺ DC number may account for the defect in their migration in CCR2^{-/-} mice, because the thymic Sirp α ⁺ cDC subset is presumed to migrate from the bloodstream (14). Most CD11c⁺B220⁻ DCs in peripheral blood and bone marrow expressed abundantly Sirp α (supplemental Fig. S4), similarly as observed on thymic Sirp α ⁺ DCs, and this population expressed CCR2 (supplemental Fig. S5). CCR2^{-/-} mice exhibited a moderate reduction in CD11c⁺B220⁻ DCs in peripheral blood, but not bone marrow (Fig. 6, A and B). This suggests a possible defect in the migration of CD11c⁺B220⁻ DCs from bone marrow in CCR2^{-/-} mice. To test this possibility, bone marrow cells were induced to differentiate to DCs with in vitro GM-CSF stimulation, labeled with CMFDA, and injected into bone marrow of WT mice (Fig. 6C, upper illustration). Under these conditions, >80% of injected cells expressed CD11c, Sirp α , and CCR2, but not B220 (supplemental Figs. S4 and S5). WT-derived DCs appeared in peripheral blood rapidly within 2 h after

the intra-bone marrow injection, whereas CCR2^{-/-} mouse-derived DCs migrated into peripheral blood less efficiently (Fig. 6, C and D). Interestingly, CD11c⁺B220⁻Sirp α ⁺ DCs appeared in thymus by 6 h after intra-bone marrow injection (Fig. 6E). These observations suggest that CCR2-mediated signals were critical of the migration of Sirp α ⁺ DCs from bone marrow into the thymus. Moreover, Sirp α ⁺ DCs were markedly decreased in PVRs of CCR2^{-/-} thymus compared with those of WT thymus (WT mice, 7.0 \pm 2.6/site; CCR2^{-/-} mice, 2.3 \pm 1.5/site; Fig. 3, D–F). Furthermore, the decrease was more evident in the region inside the PVRs compared with that outside the PVRs (Fig. 3G). CCR2 was expressed also by a portion of intrathymic Sirp α ⁺ DCs, but not Sirp α ⁻ DCs (Fig. 3H). Three mouse chemokines, MCP-1, MCP-2, and MCP-3, can bind to CCR2 (30). Among these chemokines, only MCP-2 mRNA was constitutively expressed in thymus, particularly keratin 8-positive thymic stroma, but not CD45-positive thymocytes (Fig. 7, A and B). Moreover, MCP-2 immunoreactivities were consistently detected inside the PVRs (Fig. 7C, upper panels) and on Sirp α ⁺ cells in the PVRs (Fig. 7C, lower panels). Thus, it is probable that the CCR2-MCP-2 interaction can contribute to intrathymic localization of Sirp α ⁺ DCs, particularly in the PVRs.

Defective Ag uptake by Sirp α ⁺ DCs in CCR2^{-/-} mice

Because the PVR was proved to be a main location of the uptake of circulating Ags, we further examined the effects of CCR2

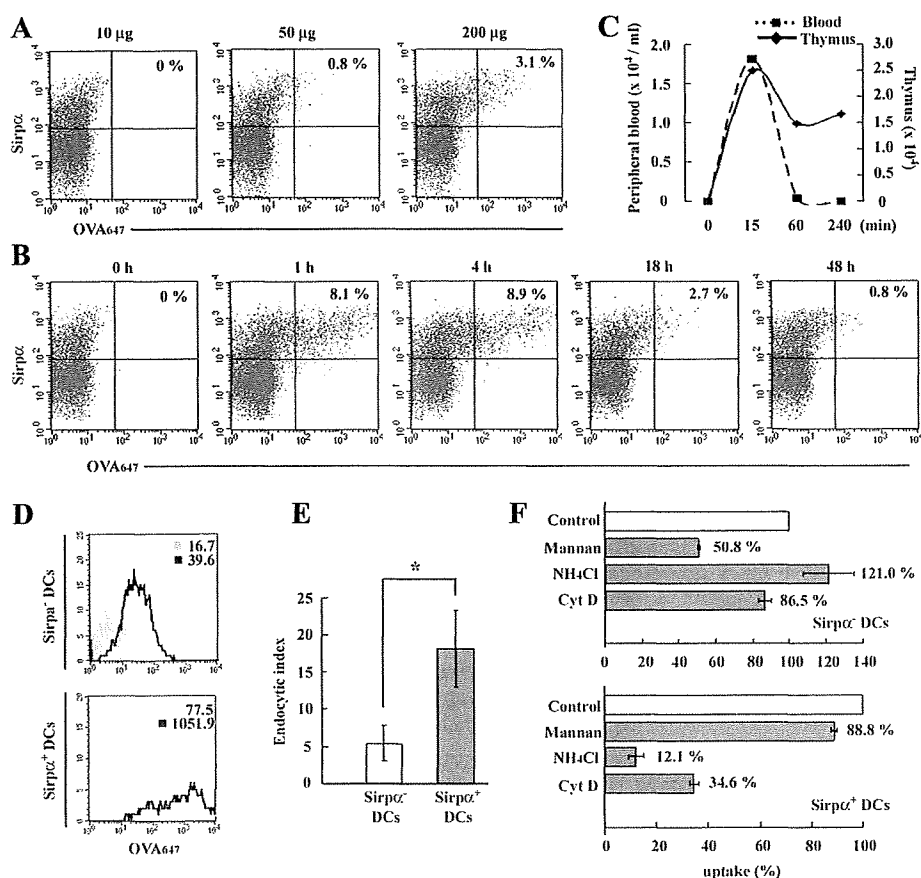


FIGURE 4. Uptake of bloodstream Ag by thymic Sirpα⁺ DCs. *A*, At 18 h after injection with OVA₆₄₇ at the indicated doses, low-density cells were isolated from WT thymus and were stained with anti-CD11c and anti-Sirpα mAbs. Then the uptake of OVA₆₄₇ in the CD11c^{high} DC population was analyzed. *B*, Uptake of OVA₆₄₇ at the indicated time points. OVA₆₄₇ (200 μg) was injected into the tail vein. Percentage of Sirpα⁺OVA₆₄₇⁺ region is shown in each panel of *A* and *B*. Representative results from three independent experiments are shown. *C*, Time kinetics of the numbers of DCs capturing OVA protein in the peripheral blood (broken line) and thymus (solid line). *D*, In vitro endocytosis of OVA₆₄₇ by CD11c^{high}Sirpα⁻ and CD11c^{high}Sirpα⁺ cDCs are shown in the upper and lower panels, respectively. Gray-filled and black-open histograms indicate the results obtained when the cells were incubated at 0 and 37°C, respectively. Numbers in each panel indicates mean fluorescence intensity for OVA₆₄₇ captured. Representative results from three independent experiments are shown here. *E*, Endocytic index in Sirpα⁻ and Sirpα⁺ cDCs. Endocytic index was calculated as mean fluorescence intensity at 37°C/mean fluorescence intensity at 0°C. Mean ± SD were calculated from three independent experiments and are shown here. *, *p* < 0.01. *F*, The effects of various agents on endocytosis. Uptake in the presence of each inhibitor is shown as the percentage of total uptake in the absence of any inhibitors. Means were calculated from three independent experiments and are shown here.

deficiency on the capability of Sirpα⁺ DCs to uptake Ags from the bloodstream. Indeed, when OVA₆₄₇ was injected i.v., CCR2^{-/-} mice exhibited a reduced proportion of intrathymic DCs capturing OVA protein compared with WT mice (Fig. 8, *A* and *B*). Moreover, after the OVA₆₄₇ injection, Sirpα⁺ DCs of WT mice contained a substantial proportion of OVA^{high} cells, which represent the cells with a higher uptake of OVA protein, and this population was markedly reduced in CCR2^{-/-} mice (Fig. 8, *C* and *D*). Moreover, among Sirpα⁺ DCs, the CCR2-expressing population was a main cell type which captured OVA protein (Fig. 8*E*). CMFDA-labeled WT mouse-derived bone marrow cells appeared in thymus 2 days after the adoptive transfer to CCR2-deficient mice and a substantial proportion of these stained cells expressed CD11c and Sirpα simultaneously (Fig. 8*F*). Sirpα⁺CD11c⁺ DCs appeared in thymus similarly when CMFDA-labeled CCR2-deficient mouse-derived bone marrow cells were adoptively transferred (data not shown). When OVA₆₄₇ was injected i.v. 2 days after the adoptive transfer, WT donor-derived Sirpα⁺CD11c⁺ DCs captured OVA protein more efficiently than CCR2-deficient DCs in the CCR2-deficient thymus (Fig. 8*G*). Thus, CCR2-mediated signals may at

least partially regulate the function of Sirpα⁺ DCs to uptake Ag from the bloodstream (supplemental Fig. S6).

Accumulation of autoreactive T cells against serum Ags in the periphery of CCR2^{-/-} mice

We observed that CCR2^{-/-} mice did not exhibit any signs suggestive of autoimmune disorders until 1 year after the birth (our unpublished data). Hence, we examined whether autoreactive T cells against certain self-Ags in the bloodstream accumulated in the periphery of CCR2^{-/-} mice. We examined the accumulation of autoreactive T cells in the draining lymph nodes in WT mice that received CFSE-labeled WT or CCR2^{-/-} mouse-derived splenocytes and were subsequently immunized with mouse serum emulsified in CFA. Immunization with total serum protein increased the cell division of CCR2^{-/-} mouse-derived CD4⁺ T cells inside draining lymph nodes (10.6%) to a greater extent than immunization with PBS (4.3%; Fig. 9*A*). Moreover, CD4⁺ T cell division was significantly increased in the recipients of CCR2^{-/-} mouse-derived splenocytes compared with the recipients of WT mouse-derived splenocytes (Fig. 9*B*). Thus, the lack of CCR2 can

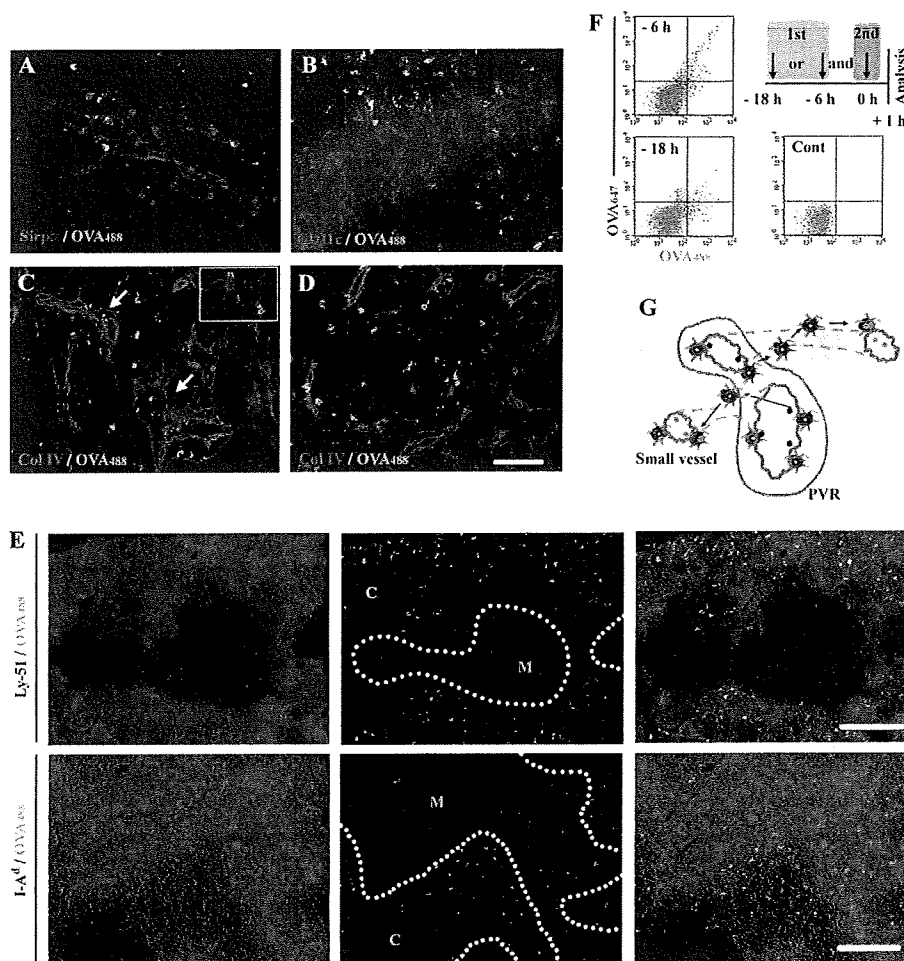


FIGURE 5. Localization of i.v. injected Ag in thymus. *A–C*, Thymic tissues were obtained 0.5 h after injection of OVA₄₈₈ and were stained to obtain a double-color fluorescent image with the combination of OVA₄₈₈ (green) and (A) Sirp α (red), (B) CD11c (red), or (C) Col IV (red). Arrows in *C* indicate the cells with captured OVA₄₈₈ inside the PVRs. The image showing the cells capturing OVA₄₈₈ in close proximity to small vessels is an *inset* in *C*. *D*, A double-color fluorescent image for OVA₄₈₈ (green) and Col IV (red) at 6 h after OVA injection. Scale bars, 100 μ m. *E*, A double-color fluorescent image with the combination of OVA₄₈₈ (green) and Ly51 (red) or I-A^d (red) at 18 h after injection is shown in the *upper* and *lower panels*, respectively. The merged images are shown in the *right panels*. Dashed lines indicate the boundary between cortex (C) and medulla (M). Scale bars, 200 μ m. *F*, OVA₆₄₇ and OVA₄₈₈ were i.v. injected consecutively with an interval of either 6 or 18 h, illustrated in *upper right panel* in *F*. Uptake of OVA protein by CD11c^{high} DC population isolated after double injection with an interval of either 6 or 18 h is shown in the *left upper and lower panels*, respectively. Autofluorescence for each parameter in the CD11c^{high} DC population without injection is shown as a control. Representative results from three independent experiments are shown here. *G*, Presumed intrathymic trafficking modes of Sirp α ⁻ DCs, combined with the Ag uptake. Blue and green particles indicate OVA₆₄₇ and OVA₄₈₈, respectively.

result in enhanced accumulation of autoreactive T cells against serum self-Ags.

Discussion

Mouse thymus CD11c⁺ cDCs can be classified into two populations, a major CD8 α ⁺ and a minor CD8 α ⁻ one (31). CD8 α ⁻ cDCs can pick up CD8 $\alpha\beta$ heterodimer from thymocytes and retain them on the cell surface, thus precluding the use of CD8 α as a reliable marker to distinguish these two populations. Wu and Shortman (8) observed that CD8 α ⁻ but not CD8 α ⁺ cDCs simultaneously express the Sirp α molecule and proposed the use of Sirp α as a marker of this minor cDC population. Concomitantly, it was proposed that the interaction between thymocytes and DCs in thymic cortex can also have profound effects on positive selection (32). Likewise, McCaughy et al. (33) observed that clonal deletion of autoreactive thymocytes requires the stimuli from rare CD11c⁺ cortical DCs. Given the unique localization of Sirp α ⁺

DCs confined to the cortex, these observations suggest the potential involvement of Sirp α ⁺ DCs in central tolerance, but their small number hinders the isolation for a detailed analysis of Sirp α ⁺ DC function.

A partial but selective reduction in intrathymic Sirp α ⁺ cDCs in CCR2^{-/-} mice prompted us to investigate the thymic selection process in WT and CCR2^{-/-} mice to elucidate the role of intrathymic Sirp α ⁺ cDCs in the process. When DO11.10 TCR-transgenic mice were administered immunogenic OVA_{323–339} peptide i.v., CCR2 gene ablation partially attenuated the clonal negative deletion by apoptosis of the DO11.10⁺ DP thymocyte population. Intraperitoneal injection of anti-CD3 Ab deleted thymocytes to similar extents in WT and CCR2^{-/-} mice, excluding the possibility that CCR2 deficiency impaired the apoptotic response of thymocytes. Negative selection can be exerted by various types of APCs including Sirp α ⁻ cDCs, B cells, macrophages, cortical thymic epithelial cells, and mTEC in addition to Sirp α ⁺ cDCs. We

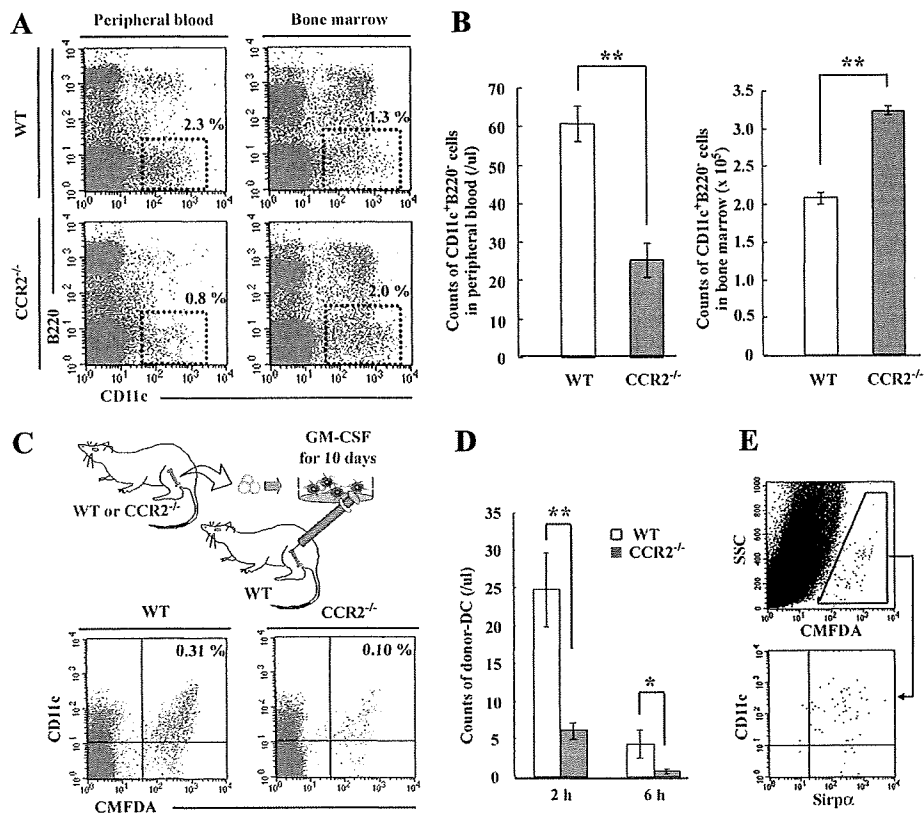


FIGURE 6. Mobilization of $\text{Sirp}\alpha^+$ DCs from bone marrow. **A** and **B**, PBMCs and bone marrow cells isolated from femur bone marrow were stained with anti-CD11c and anti-B220 mAbs. Proportion (**A**) and the numbers (**B**) of $\text{CD11c}^+\text{B220}^-$ cells gated with the dot squares were determined on peripheral blood and bone marrow in $\text{CCR2}^{-/-}$ and WT mice. Percentage of gated cells is shown in each panel of **A**. Mean \pm SD were calculated from three independent experiments and are shown here. **C**, The image of experimental procedure of “trafficking of bone marrow-derived DCs” was illustrated and is shown in the *upper panel*. PBMCs were isolated from the recipients 2 h after injection and stained with anti-CD11c mAb. Egress of $\text{CCR2}^{-/-}$ bone marrow-derived DCs into peripheral blood was compared with WT DCs. Percentage of donor DCs in $\text{CMFDA}^+\text{CD11c}^+$ region is shown in each panel. **D**, The numbers of donor-derived DCs in peripheral blood were determined 2 and 6 h after injection. Mean \pm SD calculated from five independent experiments are shown here. *, $p < 0.05$ and **, $p < 0.01$. **E**, One $\times 10^7$ WT bone marrow cell-derived DCs were injected into both the right and left tibial cavity. Six hours after injection, expression of $\text{Sirp}\alpha$ and CD11c on intrathymic migrated CMFDA^+ donor cells was analyzed by FCM. Representative results from four independent experiments are shown here.

failed to detect any apparent differences in other APC populations than $\text{Sirp}\alpha^+$ cDCs between WT and $\text{CCR2}^{-/-}$ mice. Thus, it is unlikely that reduced negative selection in $\text{CCR2}^{-/-}$ mice can be

ascribed to the changes in these cell populations. Furthermore, accumulating evidence implicates intrathymic $\text{CD4}^+\text{CD25}^+$ regulatory T cells as an essential cell component in central tolerance.

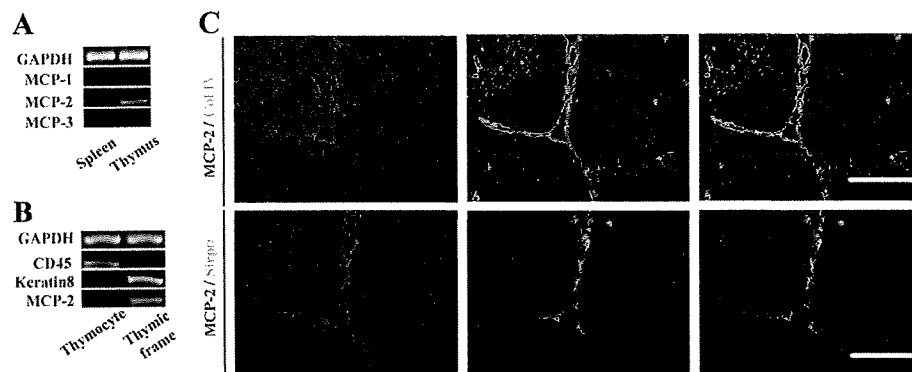


FIGURE 7. Expression of CCR2 ligands in thymus under physiological condition. **A**, Total RNAs were extracted from thymus and spleen of WT mice. Expression of CCR2 ligands, MCP-1, MCP-2, and MCP-3, was determined by RT-PCR. GAPDH served as an internal positive control. **B**, Thymic tissues were mechanically disrupted and fractionated into thymocyte and thymic stromal components. MCP-2 transcripts were determined on these two fractions by RT-PCR. CD45 and keratin 8 served as positive control for the thymocyte and thymic stromal fraction, respectively. **C**, Double-color fluorescent immunostaining for MCP-2 (red) and Col IV (green) or MCP-2 (red) and $\text{Sirp}\alpha$ (green) in the thymic tissue sections are shown in the *upper* and *lower panels*, respectively. The merged images are shown in the *right panels*. Representative results from three independent animals are shown here. Scale bars, 100 μm .

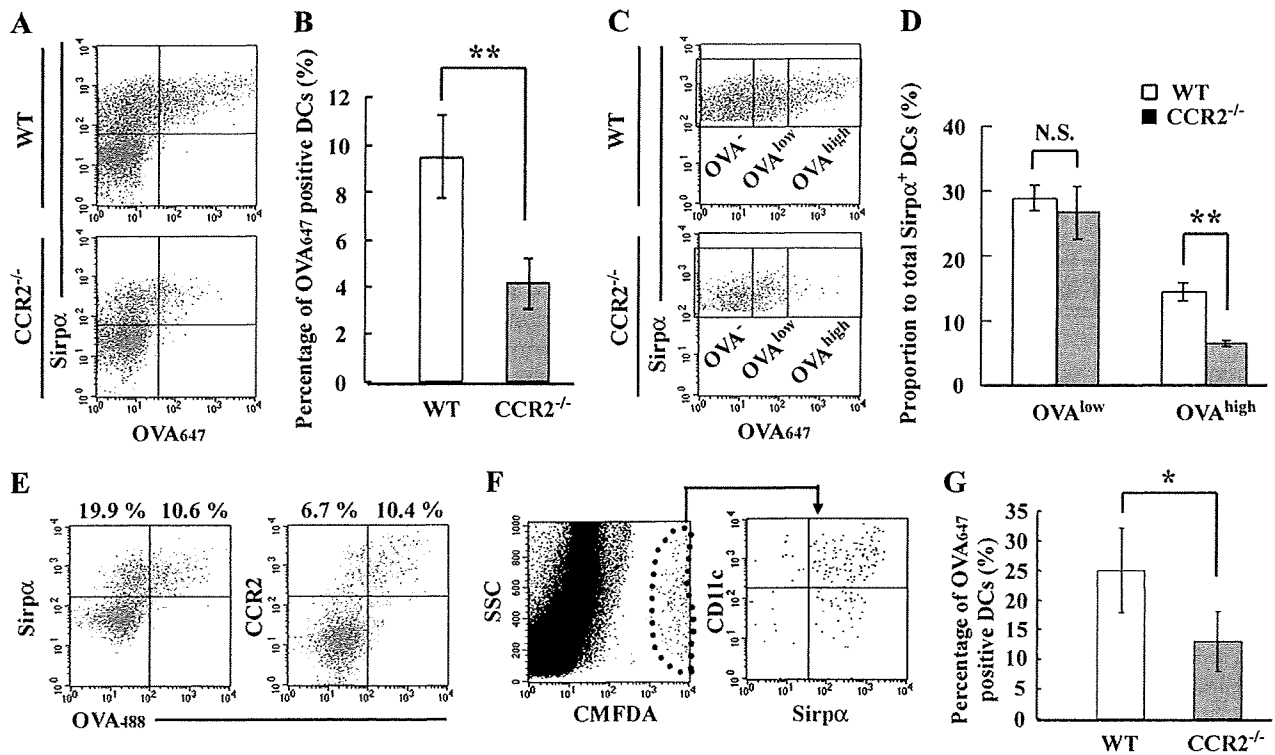


FIGURE 8. Effects of CCR2 deficiency on Ag uptake by thymic Sirpα⁺ DCs. *A*, The uptake of OVA₆₄₇ in the CCR2^{-/-} CD11c^{high} DC population at 4 h after i.v. injection was compared with WT-derived cells. *B*, Percentage of DCs capturing OVA₆₄₇ in the CD11c^{high} DC population. Mean ± SD were calculated from five independent experiments and are shown here. **, *p* < 0.01. *C*, Sirpα⁺ DCs derived from WT and CCR2^{-/-} thymus were separated into three groups according to the efficiency of OVA₆₄₇ uptake, OVA⁻; DCs without capturing OVA₆₄₇, OVA^{low}; DCs capturing OVA₆₄₇ with a low efficiency, and OVA^{high}; and DCs capturing OVA₆₄₇ with a high efficiency. *D*, Percentage of OVA^{low} and OVA^{high} in WT and the CCR2^{-/-} Sirpα⁺ DC population. Mean ± SD were calculated from five independent experiments and are shown here. **, *p* < 0.01; N.S., no significant difference. *E*, OVA₄₈₈ was i.v. injected into WT mice. One hour after injection, low-density cells were stained with anti-CD11c and anti-Sirpα or anti-CCR2 mAbs. The uptake of OVA₄₈₈ and expression of Sirpα or CCR2 in the CD11c^{high} DC population are shown. Percentage of Sirpα⁺OVA₄₈₈⁺ and OVA₄₈₈⁻, or CCR2⁺OVA₄₈₈⁻ and OVA₄₈₈⁻ regions are shown in the left or right panel. Representative results from three independent experiments are shown here. *F*, Migration of Sirpα⁺ DCs into the thymus at 2 days after i.v. injection of CMFDA-labeled WT bone marrow cells into CCR2^{-/-} mice. Expression of CD11c and Sirpα on CMFDA⁺ donor-derived cells is shown in the right panel. Representative results from three independent experiments are shown here. *G*, OVA₆₄₇ was i.v. injected into CCR2^{-/-} mice at 2 days after injection of bone marrow cells. Percentage of WT and CCR2^{-/-} donor-derived DCs capturing OVA₆₄₇ in the CMFDA⁺CD11c^{high} region are shown. Mean ± SD were calculated from four independent experiments and are shown here. *, *p* < 0.05.

Indeed, Proietto et al. (14) recently reported the capability of Sirpα⁺ cDCs to induce the differentiation of regulatory T cells in vitro. However, OVA peptide injection induced the differentiation of regulatory T cells to similar extents in both DO11.10 and DO11.10/CCR2^{-/-} thymus. Thus, it is probable that CCR2 deficiency reduced modestly intrathymic Sirpα⁺ DCs without affecting regulatory cell induction and partially attenuated negative selection in vivo.

It remains elusive on the trafficking modes of Sirpα⁺ DCs. In CCR2^{-/-} mice, Sirpα⁺ DCs were decreased moderately in peripheral blood and thymus, but were increased in bone marrow. Considering that CCR2 signaling can regulate the mobilization of monocytes from bone marrow to peripheral blood (34, 35), these observations raised the possibility of a defect in the trafficking of Sirpα⁺ DCs from bone marrow in CCR2^{-/-} mice. Indeed, WT mouse-derived Sirpα⁺ DCs, injected into bone marrow, appeared first in peripheral blood and then the thymus. On the contrary, CCR2^{-/-} mouse-derived Sirpα⁺ DCs exhibited impairment in the egress from bone marrow to peripheral blood. These observations suggest that bone marrow-derived Sirpα⁺ DCs migrated to peripheral blood in response to CCR2-mediated signals and subsequently traffic to the thymus.

In the thymus, Sirpα⁺ DCs were characteristically localized in close proximity to small blood vessels and inside the PVRs, sites which are compartmentalized by a vascular basement membrane and a border membrane separating them from the thymic parenchyma (36). It is of note that Sirpα⁺ cells in the PVRs were markedly decreased in CCR2^{-/-} mice to a greater extent than the decrease in total Sirpα⁺ cell number. Thus, intrathymic CCR2 signaling can regulate their unique localization. This notion was supported by the observation that MCP-2, a potential ligand for CCR2, was constitutively detected in the PVRs, where Sirpα⁺ DCs were present.

PVRs can provide a pathway for hematopoietic progenitor cells and mature T cells to traverse from the bloodstream to the thymic parenchyma (36) and are presumed to constitute the blood-thymus barrier, which can protect the thymic parenchyma from bloodstream-derived macromolecules (28). Thus, the unique localization of Sirpα⁺ cDCs in the thymus suggested their potential interactions with bloodstream-derived Ag. This assumption was strengthened by our present observation that intrathymic Sirpα⁺ cDCs rapidly and specifically captured OVA protein and serum IgG following i.v. injection. Moreover, injected Ags were initially detected inside PVRs or in nearby small vessels and were subsequently in the cortical parenchyma, and the injected Ag-derived

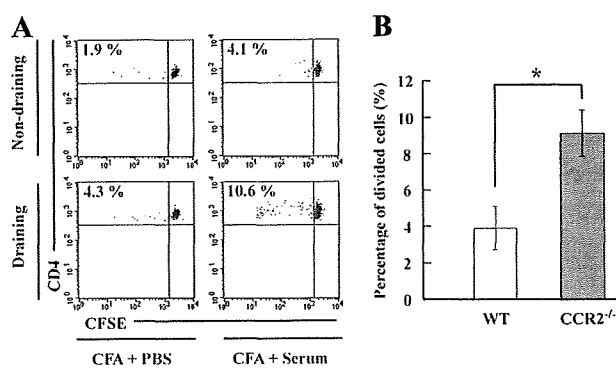


FIGURE 9. Accumulation of autoreactive T cells against serum Ags in the spleen. Spleen mononuclear cells were isolated from WT or $CCR2^{-/-}$ mice and i.v. injected into WT mice after labeling with CFSE. **A**, Recipients of $CCR2^{-/-}$ mouse-derived splenocytes were immunized with total mouse serum or PBS emulsified in CFA at 1 day after injection. Four days after, draining and nondraining lymph nodes were harvested and division of CFSE $^+$ donor-derived CD4 $^+$ T cells was analyzed by FCM. Representative results from three independent experiments are shown here. **B**, Percentage of divided CD4 $^+$ T cells was determined in the draining lymph nodes of the recipients of either WT-derived or $CCR2^{-/-}$ donor-derived splenocytes when the recipients were immunized with mouse serum emulsified in CFA. Mean \pm SD were calculated from three independent experiments and are shown here. *, $p < 0.01$.

signals were consistently colocalized with CD11c and Sirp α . Thus, after CD11c $^+$ Sirp α^+ cDCs, located around the PVRs, capture the Ags, they presumably move to cortical parenchyme to educate T cells. Indeed, $CCR2^{-/-}$ thymus-derived Sirp α^+ DCs exhibited a reduced capacity to uptake OVA. The lack of CCR2 can hinder the proper intrathymic localization of Sirp α^+ DCs and their distinctive function, Ag uptake from bloodstream, thereby reducing Ag presentation in the cortical parenchyme and subsequent negative selection against a blood-borne Ag. This hypothesis is supported by our observation in that CD4 $^+$ T cells reactive to certain serum self-Ags accumulated in the periphery of the recipients of $CCR2^{-/-}$ mouse-derived splenocytes to a greater extent than the recipients of WT mouse-derived splenocytes.

DCs can uptake free soluble Ags, in three distinct manners, by clathrin-mediated endocytosis, nonclathrin/caveolae endocytosis, and macropinocytosis (25). Thymic Sirp α^+ cDCs could endocytose OVA Ags more efficiently than thymic Sirp α^- cDCs when they were cultured in vitro with OVA Ags. Furthermore, NH_4Cl , an inhibitor of clathrin-mediated endocytosis (26), markedly inhibited OVA endocytosis by Sirp α^+ cDCs, but not by Sirp α^- cDCs. On the contrary, OVA protein endocytosis by Sirp α^- DCs was partially inhibited by mannan, whereas mannan had few effects on OVA protein endocytosis by Sirp α^+ DCs. These observations suggest that thymic Sirp α^+ cDCs characteristically can efficiently endocytose Ags in a manner distinct from thymic Sirp α^- cDCs.

Balazs et al. (29) reported that bloodstream DCs could efficiently capture and transport particulate bacteria into the spleen when particulate bacteria were i.v. injected. We also observed that CD11c $^+$ DCs rapidly disappeared from peripheral blood after uptake of i.v. injected OVA protein. Given the capacity of CD11c $^+$ DCs to move rapidly from blood to thymus, blood CD11c $^+$ DCs may migrate into thymus after capturing the i.v. injected Ag. However, Ag-capturing DCs appeared very rapidly in the thymus, reaching maximal levels before disappearance of Ag-capturing circulating DCs from the peripheral blood. Furthermore, when OVA protein was injected i.v. into mice that contained bloodstream DCs

labeled with fluorescent-conjugated latex beads, latex-labeled DCs did not appear in the thymus (our unpublished data). Thus, it is remotely possible that bloodstream DCs captured OVA protein and subsequently migrated into thymus.

In this study, we identified the unique intrathymic localization and functions of thymic Sirp α^+ DCs that are involved in negative selection, particularly against blood-borne Ags. Serum protein can also induce negative selection in thymus (27, 37) but the molecular and cellular mechanisms remain to be elucidated. Because Sirp α^+ cDCs can uptake serum protein such as IgG, these cells may induce central tolerance to blood-borne-derived Ags, in addition to Ags presented by the well-characterized intrathymic AIRE-mediated pathway.

We have shown that CCR2-mediated signals can regulate various biological aspects of Sirp α^+ DCs such as their appropriate intrathymic localization and Ag uptake capacity. It is widely held that CCR2 might be a potential therapeutic target for several autoimmune disorders. However, because CCR2-mediated signals may contribute to thymic negative selection against blood-borne Ags, CCR2 blockade may aggravate autoimmune disorders similar to the observation on the murine collagen-induced arthritis model (38). Moreover, Lauritszen et al (39) reported that proteins secreted from tumor cells into peripheral blood were transported into the thymus to eventually cause clonal deletion of tumor Ag-specific T cell repertoires. Given the potential capacity of intrathymic Sirp α^+ DCs to capture blood-borne Ags, they may have a role in the development of tumor tolerance. Because human thymus contains DCs with similar phenotypes and intrathymic localization as Sirp α^+ cDCs (40), a more detailed elucidation of the functions of Sirp α^+ cDCs may provide us with useful insights to develop a better therapeutic strategy for cancer and stem cell transplantation as well as autoimmune disorders.

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Disclosures

The authors have no financial conflict of interest.

References

1. von Boehmer, H., I. Aifantis, F. Gounari, O. Azogui, L. Haughn, I. Apostolou, E. Jackel, F. Grassi, and L. Klein. 2003. Thymic selection revisited: how essential is it? *Immunol. Rev.* 191: 62–78.
2. Anderson, M. S., E. S. Venanzi, Z. Chen, S. P. Berzins, C. Benoist, and D. Mathis. 2005. The cellular mechanism of Aire control of T cell tolerance. *Immunity* 23: 227–239.
3. Anderson, M. S., E. S. Venanzi, L. Klein, Z. Chen, S. P. Berzins, S. J. Turley, H. von Boehmer, R. Bronson, A. Dierich, C. Benoist, and D. Mathis. 2002. Projection of an immunological self shadow within the thymus by the aire protein. *Science* 298: 1395–1401.
4. Liston, A., S. Lesage, J. Wilson, L. Peltonen, and C. C. Goodnow. 2003. Aire regulates negative selection of organ-specific T cells. *Nat. Immunol.* 4: 350–354.
5. Anderson, G., K. M. Partington, and E. J. Jenkinson. 1998. Differential effects of peptide diversity and stromal cell type in positive and negative selection in the thymus. *J. Immunol.* 161: 6599–6603.
6. Marrack, P., D. Lo, R. Brinster, R. Palmiter, L. Burkly, R. H. Flavell, and J. Kappler. 1988. The effect of thymus environment on T cell development and tolerance. *Cell* 53: 627–634.
7. Matzinger, P., and S. Guerder. 1989. Does T-cell tolerance require a dedicated antigen-presenting cell? *Nature* 338: 74–76.
8. Wu, L., and K. Shortman. 2005. Heterogeneity of thymic dendritic cells. *Semin. Immunol.* 17: 304–312.
9. Liu, Y. J. 2006. A unified theory of central tolerance in the thymus. *Trends Immunol.* 27: 215–221.
10. Bendriss-Vernere, N., C. Barthelemy, I. Durand, C. Bruand, C. Dezutter-Dambuyant, N. Mouliau, S. Berrih-Aknin, C. Caux, G. Trinchieri, and F. Briere. 2001. Human

- thymus contains IFN- α -producing CD11c⁻, myeloid CD11c⁺, and mature interdigitating dendritic cells. *J. Clin. Invest.* 107: 835–844.
11. Sprent, J., and S. R. Webb. 1995. Intrathymic and extrathymic clonal deletion of T cells. *Curr. Opin. Immunol.* 7: 196–205.
 12. Heino, M., P. Peterson, N. Sillanpaa, S. Guerin, L. Wu, G. Anderson, H. S. Scott, S. E. Antonarakis, J. Kudoh, N. Shimizu, et al. 2000. RNA and protein expression of the murine *autoimmune regulator gene (Aire)* in normal, RelB-deficient and in NOD mouse. *Eur. J. Immunol.* 30: 1884–1893.
 13. Kyewski, B., and J. Derbinski. 2004. Self-representation in the thymus: an extended view. *Nat. Rev. Immunol.* 4: 688–698.
 14. Proietto, A. I., S. van Dommelen, P. Zhou, A. Rizzitelli, A. D'Amico, R. J. Steptoc, S. H. Naik, M. H. Lahoud, Y. Liu, P. Zheng, et al. 2008. Dendritic cells in the thymus contribute to T-regulatory cell induction. *Proc. Natl. Acad. Sci. USA* 105: 19869–19874.
 15. Heinzel, K., C. Benz, and C. C. Bleul. 2007. A silent chemokine receptor regulates steady-state leukocyte homing in vivo. *Proc. Natl. Acad. Sci. USA* 104: 8421–8426.
 16. Kim, C. H. 2005. The greater chemotactic network for lymphocyte trafficking: chemokines and beyond. *Curr. Opin. Hematol.* 12: 298–304.
 17. Schutyser, E., A. Richmond, and J. Van Damme. 2005. Involvement of CC chemokine ligand 18 (CCL18) in normal and pathological processes. *J. Leukocyte Biol.* 78: 14–26.
 18. Vecchi, A., L. Massimiliano, S. Ramponi, W. Luini, S. Bernasconi, R. Bonocchi, P. Allavena, M. Parmentier, A. Mantovani, and S. Sozzani. 1999. Differential responsiveness to constitutive vs. inducible chemokines of immature and mature mouse dendritic cells. *J. Leukocyte Biol.* 66: 489–494.
 19. Niess, J. H., S. Brand, X. Gu, L. Landsman, S. Jung, B. A. McCormick, J. M. Vyas, M. Boes, H. L. Ploegh, J. G. Fox, et al. 2005. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* 307: 254–258.
 20. Gao, J. L., T. A. Wynn, Y. Chang, E. J. Lee, H. E. Broxmeyer, S. Cooper, H. L. Tiffany, H. Westphal, J. Kwon-Chung, and P. M. Murphy. 1997. Impaired host defense, hematopoiesis, granulomatous inflammation and type 1-type 2 cytokine balance in mice lacking CC chemokine receptor 1. *J. Exp. Med.* 185: 1959–1968.
 21. Combadiere, C., S. Potteaux, J. L. Gao, B. Esposito, S. Casanova, E. J. Lee, P. Debre, A. Tedgui, P. M. Murphy, and Z. Mallat. 2003. Decreased atherosclerotic lesion formation in CX3CR1/apolipoprotein E double knockout mice. *Circulation* 107: 1009–1016.
 22. Murai, M., H. Yoneyama, T. Ezaki, M. Schemm, Y. Terashima, A. Harada, H. Hamada, H. Asakura, H. Ishikawa, and K. Matsushima. 2003. Peyer's patch is the essential site in initiating murine acute and lethal graft-versus-host reaction. *Nat. Immunol.* 4: 154–160.
 23. Kuziel, W. A., S. J. Morgan, T. C. Dawson, S. Griffin, O. Smithies, K. Ley, and N. Maeda. 1997. Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. *Proc. Natl. Acad. Sci. USA* 94: 12053–12058.
 24. Brewer, J. A., O. Kanagawa, B. P. Sleckman, and L. J. Muglia. 2002. Thymocyte apoptosis induced by T cell activation is mediated by glucocorticoids in vivo. *J. Immunol.* 169: 1837–1843.
 25. Mayor, S., and R. E. Pagano. 2007. Pathways of clathrin-independent endocytosis. *Nat. Rev. Mol. Cell Biol.* 8: 603–612.
 26. Sandvig, K., S. Olsnes, O. W. Petersen, and B. van Deurs. 1987. Acidification of the cytosol inhibits endocytosis from coated pits. *J. Cell Biol.* 105: 679–689.
 27. Zal, T., A. Volkman, and B. Stockinger. 1994. Mechanisms of tolerance induction in major histocompatibility complex class II-restricted T cells specific for a blood-borne self-antigen. *J. Exp. Med.* 180: 2089–2099.
 28. Bubanovic, I. V. 2003. Failure of blood-thymus barrier as a mechanism of tumor and trophoblast escape. *Med. Hypotheses* 60: 315–320.
 29. Balazs, M., F. Martin, T. Zhou, and J. Kearney. 2002. Blood dendritic cells interact with splenic marginal zone B cells to initiate T-independent immune responses. *Immunity* 17: 341–352.
 30. Murphy, P. M., M. Baggiolini, I. F. Charo, C. A. Hebert, R. Horuk, K. Matsushima, L. H. Miller, J. J. Oppenheim, and C. A. Power. 2000. International union of pharmacology: XXII. Nomenclature for chemokine receptors. *Pharmacol. Rev.* 52: 145–176.
 31. Vremec, D., J. Pooley, H. Hochrein, L. Wu, and K. Shortman. 2000. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J. Immunol.* 164: 2978–2986.
 32. Ladi, E., T. A. Schwickert, T. Chtanova, Y. Chen, P. Herzmark, X. Yin, H. Aaron, S. W. Chan, M. Lipp, B. Roysam, and E. A. Robey. 2008. Thymocyte-dendritic cell interactions near sources of CCR7 ligands in the thymic cortex. *J. Immunol.* 181: 7014–7023.
 33. McCaughy, T. M., T. A. Baldwin, M. S. Wilken, and K. A. Hogquist. 2008. Clonal deletion of thymocytes can occur in the cortex with no involvement of the medulla. *J. Exp. Med.* 205: 2575–2584.
 34. Sawanobori, Y., S. Ueha, M. Kurachi, T. Shimaoka, J. E. Talmadge, J. Abe, Y. Shono, M. Kitabatake, K. Kakimi, N. Mukaida, and K. Matsushima. 2008. Chemokine-mediated rapid turnover of myeloid-derived suppressor cells in tumor-bearing mice. *Blood* 111: 5457–5466.
 35. Tsou, C. L., W. Peters, Y. Si, S. Slaymaker, A. M. Aslanian, S. P. Weisberg, M. Mack, and I. F. Charo. 2007. Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *J. Clin. Invest.* 117: 902–909.
 36. Mori, K., M. Itoi, N. Tsukamoto, H. Kubo, and T. Amagai. 2007. The perivascular space as a path of hematopoietic progenitor cells and mature T cells between the blood circulation and the thymic parenchyma. *Int. Immunol.* 19: 745–753.
 37. Haribhai, D., D. Engle, M. Meyer, D. Donermeyer, J. M. White, and C. B. Williams. 2003. A threshold for central T cell tolerance to an inducible serum protein. *J. Immunol.* 170: 3007–3014.
 38. Quinones, M. P., S. K. Ahuja, F. Jimenez, J. Schaefer, E. Garavito, A. Rao, G. Chenuaux, R. L. Reddick, W. A. Kuziel, and S. S. Ahuja. 2004. Experimental arthritis in CC chemokine receptor 2-null mice closely mimics severe human rheumatoid arthritis. *J. Clin. Invest.* 113: 856–866.
 39. Lauritzen, G. F., P. O. Hofgaard, K. Schenck, and B. Bogen. 1998. Clonal deletion of thymocytes as a tumor escape mechanism. *Int. J. Cancer* 78: 216–222.
 40. Passens, L. C., D. M. Fluitsma, and Y. van Kooyk. 2008. Haematopoietic antigen-presenting cells in the human thymic cortex: evidence for a role in selection and removal of apoptotic thymocytes. *J. Pathol.* 214: 96–103.

Infection of B Cells With Hepatitis C Virus for the Development of Lymphoproliferative Disorders in Patients With Chronic Hepatitis C

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Infection with hepatitis C virus (HCV) is associated with lymphoproliferative disorders, represented by essential mixed cryoglobulinemia and B-cell non-Hodgkin's lymphoma, but the pathogenic mechanism remains obscure. HCV may infect B cells or interact with their cell surface receptors, and induce lymphoproliferation. The influence of HCV infection of B cells on the development of lymphoproliferative disorders was evaluated in 75 patients with persistent HCV infection. HCV infection was more prevalent (63% vs. 16%, 14%, or 17% $P < 0.05$ for each), and HCV RNA levels were higher (3.35 ± 3.85 vs. 1.75 ± 2.52 , 2.15 ± 2.94 or 2.10 ± 2.90 log copies/100 ng, $P < 0.01$ for each) in B cells than CD4⁺, CD8⁺ T cells or other cells. Negative-strand HCV RNA, as a marker of viral replication, was detected in B cells from four of the 75 (5%) patients. Markers for lymphoproliferative disorders were more frequent in the 50 patients with chronic hepatitis C than the 32 with chronic hepatitis B, including cryoglobulinemia (26% vs. 0%, $P < 0.001$), low CH₅₀ levels (48% vs. 3%, $P = 0.012$), and the clonality of B cells (12% vs. 0%, $P < 0.01$). By multivariate analysis, HCV RNA in B cells was an independent factor associated with the presence of at least one marker for lymphoproliferation (odds ratio: 1.98 [95% confidence interval: 1.36–7.24], $P = 0.027$). Based on the results obtained, the infection of B cells with HCV would play an important role in the development of lymphoproliferative disorders. **J. Med. Virol.** 81:619–627, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: B cells; clonality; cryoglobulinemia; hepatitis C virus; lymphoproliferative disorders

INTRODUCTION

Hepatitis C virus (HCV) can induce chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [Tong et al.,

1995; Ikeda et al., 1998]. In addition, some patients infected with HCV develop proliferative disorders of lymphocytes, such as mixed cryoglobulinemia [Agnello et al., 1992; Frangeul et al., 1996; Donada et al., 1998] and B-cell non-Hodgkin's lymphoma (NHL) [Ferri et al., 1994]. Cryoglobulinemia represents the oligoclonal proliferation of B cells and occurs in 19–56% of patients infected with HCV [Mazzaro et al., 1996; Donada et al., 1998; Weiner et al., 1998; Schmidt et al., 2000], while antibody to HCV (anti-HCV) and HCV RNA are detected more frequently in the patients with non-Hodgkin's lymphoma than in the general population (30% vs. 1.3%) [Ferri et al., 1994]. On the basis of these observations, cryoglobulinemia is considered to be a marker for lymphoproliferative disorders. In addition, rheumatoid factor (RF) in high titers and hypocomplementemia (low levels of C3, C4, or CH₅₀) are regarded as immunological markers for autoimmune disease and lymphoproliferation [Ramos-Casals et al., 2005]. In the patients with Sjögren's syndrome, for instance, hypocomplementemia was closely associated with the development of lymphoma [Ramos-Casals et al., 2005].

Although an epidemiological association has been noted between HCV infection and lymphoproliferative disorders, the pathogenic mechanisms underlying it have remained unclear. HCV would infect B cells persistently, and induce somatic mutations toward propagation in them. Recently, the replication of HCV was demonstrated in a B-cell line established from a patient infected with HCV [Sung et al., 2003], and

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somatic hypermutations in the immunoglobulin genes as well as proto-oncogenes were observed in a B-cell line infected with HCV [Machida et al., 2004]. These observations suggest direct and/or indirect effects of HCV infection of B cells on the induction of lymphoproliferative disorders. HCV infection by itself or stimulation by immune complexes containing viral antigens may trigger the clonal proliferation of B cells for the development of lymphoproliferative disorders in patients with chronic hepatitis C [Agnello, 1995; Ivanovski et al., 1998].

There is some evidence for the replication of HCV in peripheral blood mononuclear cells (PBMCs) of patients who are infected with HCV persistently [Moldvay et al., 1994; Lerat et al., 1998; Zignego and Brechot, 1999], although it is not known which of the T-, B-, and other cell-types supports the replication of HCV. It may replicate at very low levels in lymphoid cells, and they may serve as an extra-hepatic reservoir; this is implicated in recurrence and persistence of HCV infection in immunosuppressed individuals [Laskus et al., 2000]. Although current antiviral therapies can eliminate HCV from the serum, it may survive in lymphocytes thereafter [Pham et al., 2004; Radkowski et al., 2005]. The efficacy of interferon therapy on the HCV infection of lymphocytes is, as yet, uncertain.

The frequency of HCV infection of peripheral blood mononuclear cells (PBMCs) was examined in 75 patients with chronic hepatitis C, and HCV RNA levels were determined in CD4⁺, CD8⁺ T-cells, B-cells, and other cells. The replication of HCV in B cells was correlated subsequently with lymphoproliferative disorders.

MATERIALS AND METHODS

Patients

During 2003 through 2006, 75 patients infected with HCV were admitted to the Showa University Hospital, including two with acute hepatitis, 50 with chronic hepatitis, two with liver cirrhosis, 16 with hepatocellular carcinoma and five with non-Hodgkin's lymphoma. The diagnosis of HCV infection was confirmed in all these patients by the detection of anti-HCV and HCV RNA in the serum. All the patients were negative for hepatitis B surface antigen (HBsAg) or antibody to human immunodeficiency virus type-1. Serving as controls were 28 healthy individuals without HCV infection and 32 patients with chronic hepatitis B who did not have liver cirrhosis, hepatocellular carcinoma or non-Hodgkin's lymphoma. Informed consent was obtained from every participant for the purpose of this study, and the study was approved by the Ethics Committee of Showa University School of Medicine.

Isolation of Lymphoid Cells

PBMCs ($2.83 \pm 1.46 \times 10^7$) were obtained from whole blood (30 ml) by the centrifugation in a Ficoll/Hypaque gradient. Beads with the affinity for CD8⁺ cells

(MicroBeads[®]; Miltenyi Biotte, Bergisch Gladbach, Germany) were added to PBMCs, and the cell suspension was mixed well, incubated for 15 min at 4°C and centrifuged at 900g for 10 min in a tube. The tube was then placed on a magnet, and the supernatant floating free cells was transferred to another tube. The pellet containing CD8⁺ cells was collected and stored at -80°C until use. CD4⁺ and CD19⁺ cells were separated from the supernatant using similar procedures. The remaining supernatant was pelleted to make the 'other cell' fraction. Each compartment of PBMCs contained $\sim 11 \times 10^5$ – 10^6 cells.

Quantitation of HCV RNA in Lymphoid Cells

HCV RNA was determined by reverse transcription-polymerase chain reaction (RT-PCR). Briefly, total RNA was extracted from each cellular compartment using Trizol[®] Reagent (Invitrogen, Carlsbad, CA). The RNA solution was stirred-up, and a portion (1 µl from the total of 50 µl) was reverse-transcribed by AMV[®] RT (Roche, Mannheim, Germany) and amplified by the single-step PCR for 40 cycles with appropriate primers (5'-CGC GCG ACT AGG AAG ACT TC-3' and 5'-ATA GAG AAA GAG CAAC CA GG-3') that are complementary to the 5'-UTR sequence. HCV RNA was determined in 100 ng of each RNA sample by the real-time RT-PCR using the primers described previously [Ito et al., 2001]; it has a detection range over 1.0–8.0 log copies. HCV RNA was recorded as positive for the samples with titers exceeding 1.0 log copies/100 ng, in order to exclude contamination of lymphoid cells with serum HCV RNA.

RT-PCR for Detecting Negative-Strand HCV RNA

Negative-strand HCV RNA was determined by the strand-specific RT-PCR. A portion of total RNA (1 µl from 50 µl) extracted from the B-cell fraction (10^5 cells) was reverse-transcribed with the sense primer (5'-AGA CAT CGG GCC AGA AGT GTC C-3') complementary to a partial NS5B sequence of negative-strand HCV RNA, and amplified by the hot-started single-step RT-PCR for 40 cycles using the GeneAmp[®] EZ rTth RNA PCR kit (Applied Biosystems, Branchburg, CA) with the same primer and an antisense primer (5'-CGT TCA TCG GTT GGG GAG CAG G-3') located downstream of it [Castillo et al., 2005]. Controls were negative- and positive-strand HCV RNA species that had been generated by *in vitro* transcription using the pCVJ4L6S plasmid [Yanagi et al., 1998]. Assays were performed in duplicate for each sample.

Serum Markers of Lymphoproliferative Disorders

Cryoglobulinemia was detected by a semi-quantitative centrifugation method. Briefly, blood samples were centrifuged at 600g for 20 min at 37°C. Sera were cooled to 4°C and left to stand for 48 h, and centrifuged again at 2,500g for 10 min at 4°C. The emergence of cryocrit at 4°C and its disappearance by warming up to 37°C for

20 min was regarded positive for cryoglobulin. RF was determined by the latex turbidimetric assay, and C4 and CH₅₀ activities by nephelometry and Mayer's method, respectively. Markers of lymphoproliferative disorders were determined in 50 patients with chronic hepatitis C and 32 patients with chronic hepatitis B who had HBsAg in the serum. None of the patients with chronic hepatitis suffered from acute hepatitis, cirrhosis or non-Hodgkin's lymphoma.

Amplification of the V_H Region in Immunoglobulin by PCR

RNA (1 µl) from PBMCs or B cells was reverse-transcribed into cDNA and amplified using the GeneAmp[®] EZ rTth RNA PCR kit (Applied Biosystems) in accordance with the manufacturer's instructions. Amplification was carried out with FW1 primer (5'-AGG TGC AGC TGG A[T]GG[C] AGT C[G]T[G]G G-3') in the V_H region and hM3 primer (5'-GGA AAA GGG TTG GGG CGG AT-3') located 8 nt downstream from the start of C_H1 exon in C_µ region. PCR products were visualized by staining with ethidium bromide after they had been electrophoresed on 1% agarose gels.

Fingerprinting Assay for the Ig Gene

The clonality of B cells was examined by the fingerprinting assay specific for isotypes of the immunoglobulin (Ig) gene by the method of Ivanovski et al. [1998]. Briefly, the PCR products of the Ig-V_H gene, from 75 patients infected with HCV and 28 healthy controls, were examined for the length of the complementarity-determining region 3 (CDR3) by primer extension using a primer (hFW3; 5'-CTG AGG ACA CGG CCG TGT ATT ACT G-3') complementary to a conserved sequence in human V_H framework 3 (FW3) regions. The hFW3 primer was end-labeled with [γ -³²P]ATP (150 mCi/ml; Japan Radioisotope Association, Tokyo, Japan) using the phage T4 polynucleotide kinase (T4PNK; Takara, Shiga, Japan). The reaction mixture (18 µl), containing PCR products, 1 µM ³²P-labeled primer, 25 mM dNTP, 10× PCR buffer and 1 unit of Taq polymerase, was subjected to denaturation at 95°C for 8 min, annealing at 64°C for 1 min and extension at 72°C for 15 min. The reactant (9 µl) was separated by electrophoresis on 6% polyacrylamide gel supplemented with 6 M urea, dried and exposed onto radiographic films.

Subcloning and Sequencing of the V_H Region Gene

One PCR product from a patient infected with HCV that exhibited oligoclonal bands and another from a control subject, without displaying any clonal band on the Ig fingerprinting assay, were purified on gel-electrophoresis and sub-cloned into the pCR-TOPO vector (Invitrogen, Leek, The Netherlands). After they had transformed *Escherichia coli* (DH5 α), 26 and 16 clones from the patient and control, respectively, were selected and the V_H region was sequenced using the Big Dye[®] Terminator ver.1.1 Cycle Sequencing kit (Applied Biosystems, Tokyo, Japan). Deduced amino acid sequences of the VDJ region of IgM heavy chain were aligned by the BLAST search, and analyzed for any homology among clones by the GENETYX-Mac ver.13.0 software (Genetyx, Tokyo, Japan).

Statistical Analysis

The median of continuous variables, without the normal distribution, was compared by the Mann-Whitney *U* test. Comparison of discontinuous variables was performed by the χ^2 test or Fisher's exact test. A *P* value < 0.05 was considered statistically significant. Values with the normal distribution were expressed as the mean \pm SD. Data of variables, not distributed normally, were transformed into log values as required. Logistic regression modeling was used in the multivariate analysis for association with lymphoproliferative disorders with the JMP ver. 7 software (SAS Institute, Cary, NC).

RESULTS

Detection of HCV RNA in B Cells

Table I compares the detection of HCV RNA in various cellular compartments. HCV RNA was detected more frequently in B cells than CD4⁺, CD8⁺ T cells or other cells from the 75 patients (63% vs. 16%, 14%, or 17%, *P* < 0.05 for each). There were no significant differences in the detection of HCV RNA in lymphoid cells between the patients with chronic hepatitis and those with hepatocellular carcinoma or non-Hodgkin's lymphoma. HCV RNA was detected in B cells from 55 (73%) patients by the conventional RT-PCR at screening. Eight of them, with HCV RNA titers < 1.0 log copies/100 ng RNA by the real-time RT-PCR, were deemed negative for HCV RNA

TABLE I. Frequency of HCV RNA in Diverse Cell Compartments From Patients Infected With HCV

Cell types	Total (n = 75)	Chronic hepatitis ^a (n = 54)	Non-Hodgkin lymphoma (n = 5)	Hepatocellular carcinoma (n = 16)
CD8 ⁺ T cells	12 (16%)*	6 (11%)*	2 (40%)	4 (25%)
CD4 ⁺ T cells	11 (15%)*	6 (11%)*	3 (60%)	2 (13%)
B cells	47 (63%)	32 (59%)	3 (60%)	12 (75%)
Others	17 (23%)*	11 (20%)**	3 (60%)	3 (19%)

^aTwo patients each with acute hepatitis and cirrhosis without hepatocellular carcinoma were included.

*Significantly lower than the detection in B cells (*P* < 0.05).

**Significantly lower than the detection in B cells (*P* < 0.01).

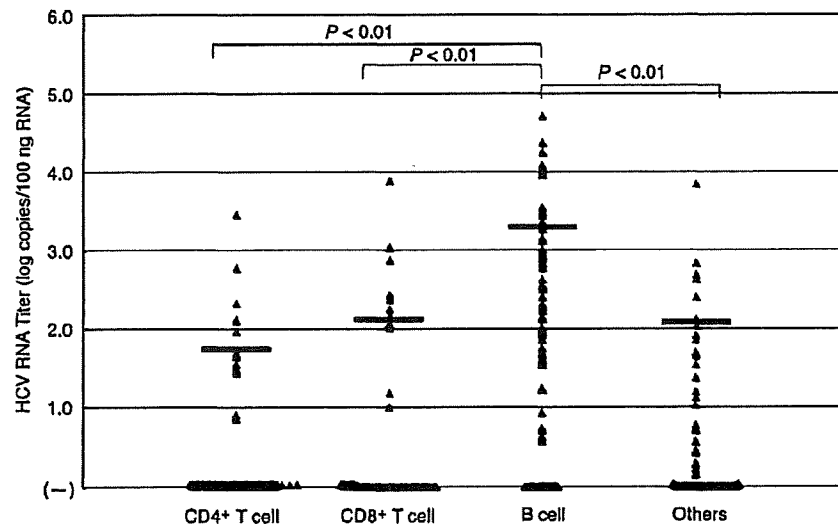


Fig. 1. HCV RNA titers in various compartments of lymphoid cells. Bold bars indicate mean values.

in order to exclude contamination by serum HCV RNA. The distribution of HCV RNA titers in various cell compartments is illustrated in Figure 1. HCV RNA levels were higher in B cells than in CD4⁺, CD8⁺ T cells or other cells (3.35 ± 3.85 vs. 1.75 ± 2.52 , 2.15 ± 2.94 or 2.10 ± 2.90 log copies/100 ng, $P < 0.01$ for each).

Replication of HCV in B Cells

A method for detecting minus-strand HCV RNA was developed (see Materials and Methods Section). It could detect negative-strand HCV RNA specifically with a sensitivity of 3.0 log copies/ml (range: 3.0–6.0 log copies/ml) without a self-priming of positive strands in control transcripts (Fig. 2A). None of the 75 patients had >7.0 log copies of HCV RNA in B cells (Fig. 1), thereby indicating the capability of this method to detect negative-strand HCV RNA in the patients studied.

Since this assay could not detect <3.0 log copies/ml of negative-strand HCV RNA, which were present in lower titers than positive strands usually, only the 16 patients with HCV RNA in B cells in titers ≥ 3.0 log copies/ml were analyzed. Negative-strand HCV RNA was detected in four (5%) of the 75 patients, indicating that HCV replicated efficiently in the B cells (lanes 2, 14, 15, and 17 in Fig. 2B).

Markers for Lymphoproliferative Disorders in Patients With Chronic Viral Hepatitis

Table II compares the detection of markers for lymphoproliferative disorders between the 50 patients with chronic hepatitis C and the 32 patients with chronic hepatitis; they did not have acute hepatitis, liver cirrhosis, hepatocellular carcinoma or non-Hodgkin's lymphoma. Cryoglobulinemia and low CH₅₀ levels

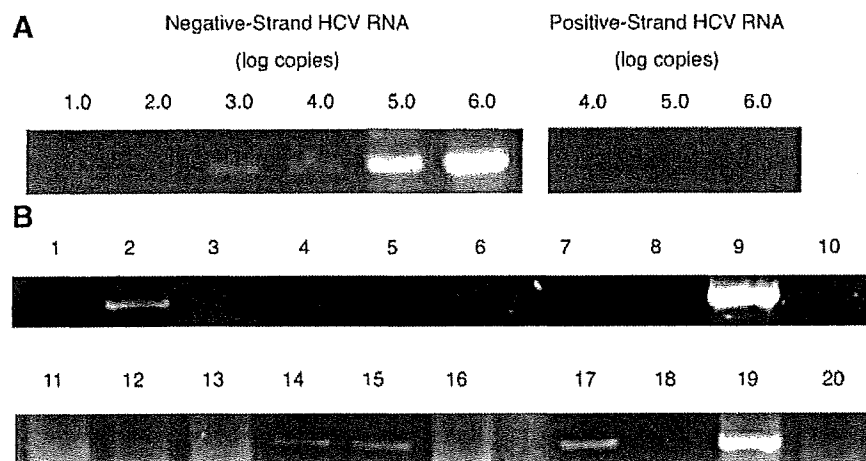


Fig. 2. A: Ethidium bromide staining of products of strand-specific RT-PCR for negative- and positive-strand HCV RNA. B: Strand specific RT-PCR for negative-strand HCV RNA from B cells of 16 patients. Positive control for negative-strand HCV RNA (lanes 9 and 19) and negative control for positive-strands HCV RNA (lanes 10 and 20) are included.

TABLE II. Markers for Lymphoproliferative Disorders in Patients Chronic Hepatitis C or B

Features ^a	Hepatitis C (n=50)	Hepatitis B (n=32)	Differences <i>P</i> value
Age (years)	52.3 ± 11.8	53.9 ± 11.9	NS
Men	32 (64%)	17 (53%)	NS
ALT (IU/L [5–25] ^b)	96.5 ± 145.9	50.7 ± 79.8	0.008
Cryoglobulinemia	13 (26%)	0 (0%)	<0.001
RF > 10 IU/ml	24 (48%)	13 (41%)	NS
C4 < 10 mg/dl	1 (2%)	1 (3%)	NS
CH ₅₀ < 20 U/ml	24 (48%)	1 (3%)	0.012
Clonality	6 (12%)	0 (0%)	<0.001
Any marker for lymphoproliferative disorders	37 (74%)	13 (41%)	0.015

ALT, alanine aminotransferase; RF, rheumatoid factor; NS, not significant.

^aData are number (%) or the mean ± SD.

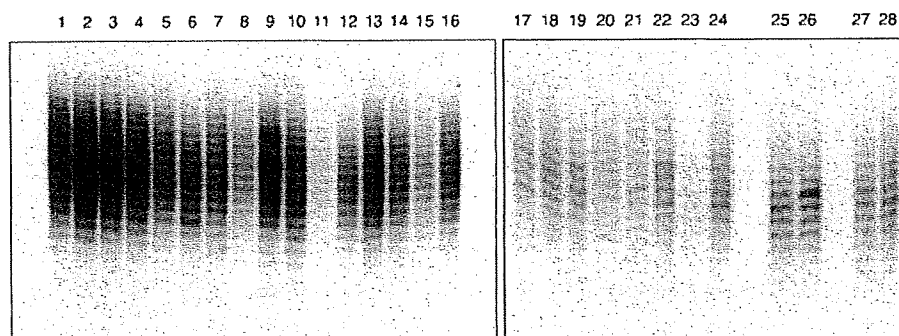
^bInterquartile normal range.

(<20 U/ml) were significantly more frequent in the patients with chronic hepatitis C than hepatitis B, while high RF titers (>10 IU/ml) were common in them both. At least one marker for lymphoproliferative disorders was detected more frequently in patients with chronic hepatitis C than hepatitis B (74% vs. 41%, *P* = 0.015). These results indicate that cryoglobulinemia and low CH₅₀ levels would be markers of lymphoproliferative disorders characteristic of the patients with chronic hepatitis C.

Oligoclonality of the Immunoglobulin Heavy Chain (Ig-V_H) Gene in Patients With HCV Infection

Fingerprinting assay of the Ig-V_H gene was performed on B cells recovered from patients infected with HCV. Ladders of PCR products from B cells of healthy controls did not produce strong bands (Fig. 3A). In contrast, the ladders of some patients with HCV infection contained a monoclonal band indicative of the oligoclonality

A Healthy Controls



B Patients with HCV Infection

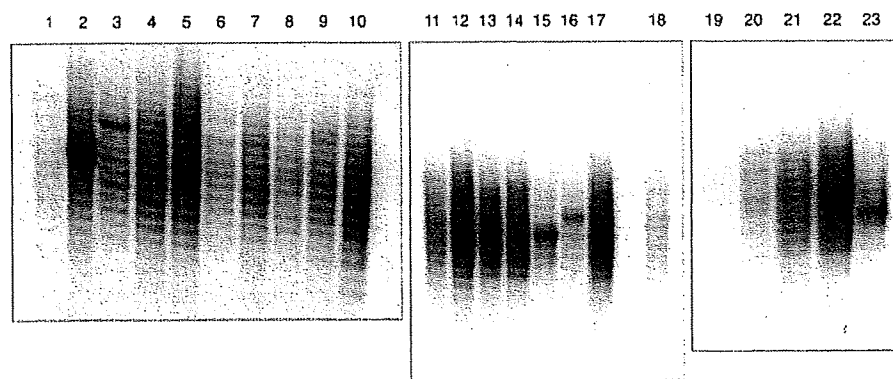


Fig. 3. Ig fingerprinting assay of (A) PBMCs from 28 healthy subjects and (B) B cells from 23 patients infected with HCV.

(Fig. 3B). The oligoclonality was observed not only in the patient with non-Hodgkin's lymphoma (lane 2 in Fig. 3B), but also in those without non-Hodgkin's lymphoma (lanes 3, 15, 16, and 23 in Fig. 3B). Overall, it was detected in B cells from 8 (11%) of the 75 patients infected with HCV, including one each with non-Hodgkin's lymphoma and hepatocellular carcinoma and six with chronic hepatitis. Six of these eight patients had B cells carrying HCV RNA, and seven possessed at least one of cryoglobulinemia (detected in three patients), high-titered RF (four patients) and hypocomplementemia (four patients). Six patients were infected with HCV group 1 and the remaining two with HCV group 2. When the same assay was undertaken on PBMCs from 32 patients with chronic hepatitis B, the oligoclonality was not observed in any of the patients (Table II).

Amino acid sequences of some clones, obtained from a patient whose Ig-V_H gene showed the oligoclonality (lane 3 in Fig. 3B), were similar (Fig. 4B). In contrast, clones obtained from a healthy subject without strong bands in Ig-V_H fingerprinting assay (lane 8 in Fig. 3A) were not similar in the sequence (Fig. 4A). These results suggest that a clonal expansion of B cells would occur in the peripheral blood of patients infected with HCV.

Factors Associated With Lymphoproliferative Disorders

Univariate analysis was performed to determine factors predisposing to lymphoproliferative disorders, including cryoglobulinemia, high-titered RF, hypocomplementemia and the B-cell clonality (Table III). Low ALT levels and the presence of HCV RNA in B cells were associated significantly with lymphoproliferative disorders. Among them, HCV RNA in B cells was the only predictive factor by multivariate analysis (odds ratio 1.98 [95% confidence interval 1.36–7.24], *P* = 0.027).

DISCUSSION

It has been demonstrated epidemiologically that HCV induces a number of extrahepatic manifestations [Cacoub et al., 1999; Zignego and Brechot, 1999], of which lymphoproliferative disorders is related most closely to HCV infection [Zignego et al., 2007]. Accordingly, it has been accepted that chronic infection with HCV can lead to the clonal expansion of B cells and that the sustained proliferation of B cells would promote the occurrence of genetic mutations. Zignego et al. [2000] have observed frequently t(14;18) translocation and overexpression of bcl-2 in lymphoid cells from patients with lymphoproliferative disorders in association with

(A) IgV_H Gene Clones from a Healthy Control

C3	GDGDRGRLAP	DVHTVVGWNA	AILQRTVIHG	RVVGSQATQL	HVGCARGCVC	GHRDSALELL	CIVCVTVISA	DPSHPLKFLP	RGLPHPADSI	AGKGVTRSPA	GDLH
C1	-----F----	---V-VVGV	-ATT-T-TS	T-IHOCVLGF	QAVHLQIGDA	FGII-*DGES	AFHRRGVFCC	PTISFASNET	YP-QPLPW-L	-NPAHSIITK	-ESR
C2	-----QSS---	G-EP-YPLRV	VPHLSRTVI	HGRVRSGHRA	RLQEGELALGR	VY*YGDSTLE	GG-V-GAPTV	IYIPNPLQSL	FWGLLDPAV	VTLDGATRS	EGEG
C13	-----Q-S---	V-KV-WASCC	YSSTVIHOCV	LCFQAVHL-I	GDAPFCIIS*D	-ESA-HRROV	FCCPTISFAS	NETY--Q---	WS-AD-H--	IT--EP--CT	-ESQ
C7	-----P----	---I---RPEP	VNPTPDECTL	IEG-ALSREA	AHL*IQ-VPA	VVSGDGESA-	HRV-IECCST	I-TNKGD-LQ	-LPWLSLTYPM	HGII-FKCESR	-CTG
C10	-----Q-S---	V-NRGGK*FQ	FGTVIHGRVL	GSQAVHLQIQ	*-LGVVSGDG	BSALHRCVCV	*-LGVVSGDG	--TTTT-NV*	N-LQ--PNSL	ADPA-VVVTE	GGPS
C12	R---HC----	-IKSIR*LPE	P-KAG-RTVI	HSRVLSQAV	-LLIQ*VLGI	VSGDGSALH	RVCV-YGTTT	TNV*NPLO	LPWLSLDPVH	-IATEGE-RG	CTGE
C16	-----F----	---V*ASON	IVSS---RS	-L----VH-	OIQ*VL-VVS	-DGE---H*V	-V-Y--TTTT	NG*D--O---	WS-ADPVA-	-TE-ESRCT	-ESC
C14	-----VQ----	V-K-*EQST	-AARNRTEVI	HGRVLSQAV	-LQIQ-VLGI	ISG-GEPA-H	GVCI-CATTT	AINN*DPLO	LPWLSLDPAH	GTAAKGESRG	CTGE
C5	-----Q-S---	QP*ISRTVIH	GCVV-SQPAQ	LHV-CADRR	PGHGDPALKL	LCIVCATTWR	VDPHPLKPL	SRGLSHPVHI	VAGEDVRSRL	--DLH	
C4	-----F-XPG-	SSQSKQLALX	HXFHSHNTRP	CPRLSQCSPA	DTVSSWR-XW	RW*IGESQSK	RSL-NYYXX*	CMKPTPA-SL	EPXGSSSCS-	H*V*IQRLHR	R-X-Q
C6	PPAARKGEFA	AAKFNSPYSE	SYNSLAVVL	QRDWNENPGV	TQLNRLAAHP	ARTDRPSQQL	RSLYVRQKVF	YPLKRESRYR	RSLYVRQKVF	LYGLVQSDII	DTPGR
C8	NQPPNLSLIS	LAVKRLICYI	**KVNTPFKT	RRFSN*IT*S	T*LYMAYRY	ALNIRLYAVP	VNGMPLH*TK	EPSPMKGVF	RYPPSQGLWQ	EYGLLL*CU*	APAAP
C11	SRATVTSKAT	AR*STTCYS	R*RSPEARA	IGIST*CRS	CPENPSTARF	PFCKLQSSI	ILCRNQQGQA	EDCIAHTCAS	VDDTRMARTF	STLRLVVAV	ICPAS
C9	-W-G*LQDKT	*SRKRYL*S	KHFT*LSFCI	YKL-IGREEX	MGRGG*GS*	LEVGSQTGIT	LKT*RSRNRG	KQTSARWQNR	LF*CF SARLH	QLH	
C15	ETVTRAPNPF	*SND--APRS	KGCLAQ*YTA	VS-AVTELSF	R-NWFLDVST	DMVTRL-REG	L*LVLAL**:	PIHSSPPFGA	AGSSSSSNY*	WSHQGC-RG	T-SPK

(B) IgV_H Gene Clones from a Patient Infected with HCV

HC17	GDGDRGRLAP	DVHTVVGWTT	IYGCRTVIHG	RVVGSQATQL	HVGCAYGHVF	GNDSFLEVL	CVVCVITAND	DPSYPLKALS	TSLSHPLHSR	TSKGBEYSLA	GDLH
HC5	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC10	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC16	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC9	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC15	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC2	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC24	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC7	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC26	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC16	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC4	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC12	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC13	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC6	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC25	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC22	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC19	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC9	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC21	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC2	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC23	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC1	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC20	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC14	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
HC11	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

Fig. 4. Aligned amino acid sequences of (A) the 16 Ig-V_H gene clones from a healthy control (lane 8 in Fig. 3A) and (B) the 26 clones from a patient infected with HCV (lane 3 in Fig. 3B). Dashes indicate the sequence identity. Three clones from the patient with more than 95% homology are boxed.