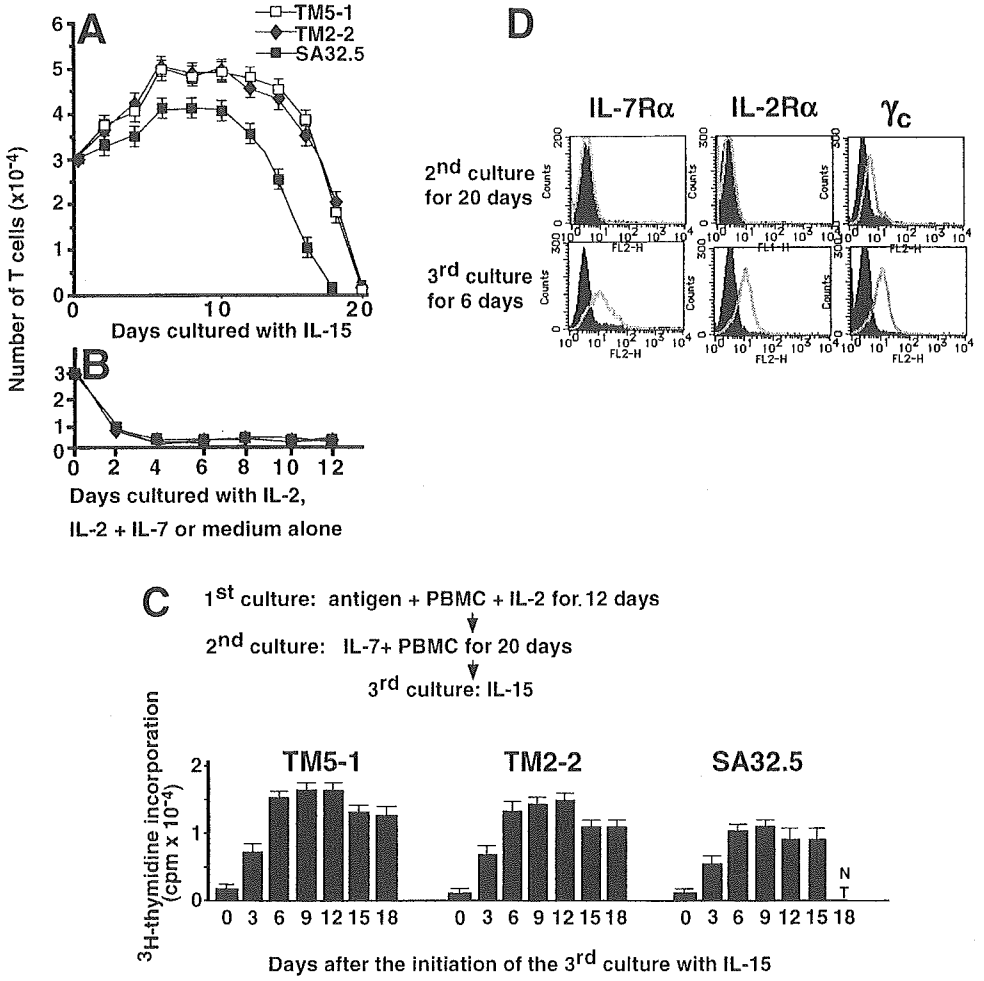

FIGURE 5 Restoration of responsiveness to antigen in recombinant human interleukin–7 (rhIL-7)–induced anergic T cells by culture with rhIL-15 but not rhIL-2. (A) Changes of T-cell number cultured with IL-15 in TM5-1 (open square), TM2-2 (solid diamond), and SA32.5 (solid square) clones. After the second culture of T cells with rhIL-7 (50 ng/ml) for 20 days, T cells were washed and cultured (the third culture) with IL-15 (100 ng/ml) for the time indicated in the abscissa. Ordinate indicates T-cell numbers. At initiation of culture, T cells (3×10^4/well) were cultured with IL-15 in a 96-well plate in triplicate. (B) Changes of T-cell numbers cultured with IL-2, IL-2 + IL-7, or medium alone in the third culture. (C) Restoration of responsiveness to antigen in IL-7–induced anergic T cells by culture with IL-15: on days 0, 3, 6, 9, 12, 15, and 18 of third culture with IL-15, respectively, T cells were harvested, washed, and assayed for proliferative response to protein purified derivative of tuberculin (10 μg/ml) in TM5-1 and TM2-2 or to glutamic acid decarboxylase (GAD)65p115-127 (10 μg/ml) in SA32.5 clone without exogenous cytokines. NT indicates not tested because collection of live cells from third cultures on day 18 was difficult for the SA32.5 clone. Largest mean value of the triplicate assay observed for T cells cultured with medium alone was 1153 cpm in all proliferative assays shown in (C). Each assay was performed in duplicate; mean values are indicated. Standard errors of mean values did not exceed 10%. (D) IL-15 induced increases in expression levels of IL-7Rα, IL-2Rα, and γC in TM5-1 T cells. On day 20 of the second culture with rhIL-7 (top) and on day 6 (bottom) of the third culture with IL-15, expression levels of IL-7Rα, IL-2Rα, and γC were measured. Solid histograms indicate cells treated with isotype-matched immunoglobulin controls.

by T cells of self-peptide/MHC complexes expressed on APCs [42]. Furthermore, purified MHC/peptide complexes and immobilized anti-CD3 antibodies were able to induce T-cell anergy [42]. Thus, MHC may be an important factor for both T-cell survival and anergy induction. In the present study, under culture conditions that used a transwell system in the second culture without T-cell–APC contact, surviving T cells were markedly decreased, indicating the importance of cell contact between T cells and PBMCs for T-cell survival (Figure 2A-b).

However, addition of a mixture of anti–HLA-DR, -DQ, and -DP antibodies in the second culture with IL-7 induced a small reduction of surviving T-cell number

compared with control, indicating besides MHC, other molecules may play more important roles. In contrast, the cell contact between T cells and PBMCs had no notable effects on the T-cell anergy induction (Figure 2B). These results indicated that the cell contacts played an important role in prompting the survival of anergic T cells. Thus, IL-7 and cell-cell contacts may exert an interdependent role in the anergy induction. We also postulated that the anergy induction might involve a change of T-cell costimulation. It was reported that CTLA4, ICOS, or PD-1 expressed on activated T cells introduced negative signals into T cells to induce T-cell anergy [34, 35]. However, anergic T cells induced by coculture with IL-7 did not express these molecules, indicating that these molecules were not involved in induction of T-cell anergy (Figure 4).

Even though lack of expression of costimulatory molecule CD40L or CD28 may play an important role in other anergy induction systems [36, 37], our anergic T cells exhibited definitive expression levels of the two molecules. Thus, the mechanism of the anergy induction by IL-7 may be different from those previously reported. The anergic T cells had completely lost expression of IL-7Rα, IL-2Rα, and γc in three T-cell clones investigated (Figure 4). An alternative model would be that the induction of anergy correlated with losses of IL-2Rα, IL-7Rα, and γc expressions. Interestingly, culture of anergic T cells in the presence of IL-15 but not IL-2 markedly restored antigen-induced T-cell proliferation, another characteristic of T-cell anergy observed in the present study. It was reported that responsiveness of anergic T cells was restored by IL-2 [36, 41]. Because characteristics of the T-cell anergy in the present study were different from those reported previously, the pathway involved in restoration from T-cell anergy may also be different from previous observations. It was surprising to us that IL-15 reversed the T-cell anergy and restored partial expressions of IL-7Rα, IL-2Rα, and γc (Figure 5). Thus, reexpression of these cytokine receptors may correlate with restoration of the T-cell responsiveness. On the other hand, IL-2 + IL-7 did not reverse the T-cell anergy (data not shown), compatible with observations that anergic T-cells did not express both IL-2R or IL-7R.

Whether function of the anergic T-cells is involved in immunosuppression is an important question. As reported, human anergic regulatory T cells could be propagated by antigen-specific stimulation in the presence of IL-2 or IL-15, and after the expansion, the anergic regulatory T cells did not lose regulatory function [43–45]. However, our results revealed that the IL-7–induced anergic T cells had no immunosuppressive function (data not shown), suggesting that the anergic phenotype of $CD4^+$ T cells does not necessarily correlate with regulatory function of $CD4^+$ T cells.

Our study for the first time demonstrates the functional relationship of cytokines IL-7 and IL-15 in induction and restoration of T-cell anergy, respectively. This may prove to be a novel mechanism for regulation of helper T-cell response to nonself antigens and induction of peripheral tolerance. If IL-7 plays an important role in the maintenance of peripheral tolerance of helper T cells to self-antigen, the cancellation of helper T-cell anergy induced by IL-15 may be one of mechanisms triggering autoimmune diseases.

ACKNOWLEDGMENTS

This work was supported in part by Grant-in-Aid 14370115 from the Ministry of Education, Science, Technology, Sports, and Culture, Japan, and a research grant for intractable diseases supported by the Ministry of Health, Labor, and Welfare, Japan. We thank Dr. C. Ejima (Ajinomoto Co., Kawasaki, Japan) for the provision of rhIL-2 and M. Ohara (Fukuoka) for helpful comments.

REFERENCES

1. Van Parijs L, Refaeli Y, Lord JD, Nelson BH, Abbas AK, Baltimore D: Uncoupling IL-2 signals that regulate T cell proliferation, survival, and Fas-mediated activation-induced cell death. Immunity 11:281, 1999.
2. Smith KA: Interleukin-2: inception, impact, and implications. Science 240:1169, 1988.
3. Miyazaki T, Liu ZJ, Kawahara A, Minami Y, Yamada K, Tsujimoto Y, Barsoumian EL, Permutter RM, Taniguchi T: Three distinct IL-2 signaling pathways mediated by bcl-2, c-myc, and lck cooperate in hematopoietic cell proliferation. Cell 81:223, 1995.
4. Akbar AN, Borthwick NJ, Wickremasinghe RG, Panayoitidis P, Pilling D, Bofill M, Krajewski S, Reed JC, Salmon M: Interleukin-2 receptor common gamma-chain signaling cytokines regulate activated T cell apoptosis in response to growth factor withdrawal: selective induction of anti-apoptotic (bcl-2, bcl-xL) but not proapoptotic (bax, bcl-xS) gene expression. Eur J Immunol 26:294, 1996.
5. Sadlack B, Merz H, Schorle H, Schimpl A, Feller AC, Horak I: Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. Cell 75:253, 1993.
6. Suzuki H, Kundig TM, Furlonger C, Wakeham A, Timms E, Matsuyama T, Schmits R, Simard JJ, Ohashi PS, Griesser H, Taniguchi T, Paige CJ, Mark TW: Deregulated T cell activation and autoimmunity in mice lacking interleukin-2 receptor beta. Science 268:1472, 1995.
7. Hofmeister R, Khaled AR, Benbernou N, Rajnavolgyi E, Muegge K, Durum SK: Interleukin-7: physiological roles

and mechanisms of action. Cytokine Grow Fact Rev 10:41, 1999.

8. de Saint-Vis B, Fugier-Vivier I, Massacrier C, Gaillard C, Vanbervliet B, Ait-Yahia S, Banchereau J, Liu YJ, Lebecque S, Caux C: The cytokine profile expressed by human dendritic cells is dependent on cell subtype and mode of activation. J Immunol 160:1666, 1998.
9. von Freeden-Jeffry U, Vieira P, Lucian LA, McNeil T, Burdach SE, Murray R: Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. J Exp Med 181:1519, 1995.
10. Peschon JJ, Morrissey PJ, Grabstein KH, Ramsdell FJ, Maraskovsky E, Gliniak BC, Park LS, Ziegler SF, Williams DE, Ware CB, Meyer JD, Davison BL: Early lymphocyte expansion is severely impaired in interleukin 7 receptor–deficient mice. J Exp Med 180:1955, 1994.
11. Puel A, Ziegler SF, Buckley RH, Leonard WJ: Defective IL7R expression in T(−)B(+)NK(+) severe combined immunodeficiency. Nat Genet 20:394, 1998.
12. Schluns KS, Kieper WC, Jameson SC, Lefrancois L: Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells *in vivo*. Nature Immunol 1:426, 2000.
13. Tan JT, Dudl E, LeRoy E, Murray R, Sprent J, Weinberg KI, Surh CD: IL-7 is critical for homeostatic proliferation and survival of naive T cells. Proc Natl Acad Sci U S A 98:8732, 2001.
14. Grabstein KH, Eisenman J, Shanebeck K, Rauch C, Srinivasan S, Fung V, Beers C, Richardson J, Schoenborn MA, Ahdieh M, Johnson L, Alderson MR, Watson JD, Anderson DM, Gill JG: Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. Science 264:965, 1994.
15. Giri JG, Anderson DM, Kumaki S, Park LS, Grabstein KH, Cosman D: IL-15, a novel T cell growth factor that shares activities and receptor components with IL-2. J Leukoc Biol 57:763, 1995.
16. Burton JD, Bamford RN, Peters C, Grant AJ, Kurys G, Goldman CK, Brennan J, Roessler E, Waldmann TA: A lymphokine, provisionally designated interleukin T and produced by a human adult T-cell leukemia line, stimulates T-cell proliferation and the induction of lymphokine-activated killer cells. Proc Natl Acad Sci U S A 91:4935, 1994.
17. Bamford RN, Grant AJ, Burton JD, Peters C, Kurys G, Goldman CK, Brennan J, Roessler E, Waldmann TA: The interleukin (IL) 2 receptor beta chain is shared by IL-2 and a cytokine, provisionally designated IL-T, that stimulates T-cell proliferation and the induction of lymphokine-activated killer cells. Proc Natl Acad Sci U S A 91:4940, 1994.
18. Carson WE, Giri JG, Lindemann MJ, Linett ML, Ahdieh M, Paxton R, Anderson D, Eisenmann J, Grabstein K, Caligiuri MA: Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. J Exp Med 180:1395, 1994.
19. Wilkinson PC, Liew FY: Chemoattraction of human blood T lymphocytes by interleukin-15. J Exp Med 181: 1255, 1995.
20. Armitage RJ, Macduff BM, Eisenman J, Paxton R, Grabstein KH: IL-15 has stimulatory activity for the induction of B cell proliferation and differentiation. J Immunol 154:483, 1995.
21. Leonard WJ: Cytokines and immunodeficiency diseases. Nat Rev Immunol 1:200, 2001.
22. Leonard WJ, Lin JX: Cytokine receptor signaling pathways. J Allergy Clin Immunol 105:877, 2000.
23. Jinushi M, Takehara T, Tatsumi T, Kanto T, Groh V, Spies T, Suzuki T, Miyagi T, Hayashi N: Autocrine/paracrine IL-15 that is required for type I IFN–mediated dendritic cell expression of MHC class I–related chain A and B is impaired in hepatitis C virus infection. J Immunol 171:5423, 2003.
24. Ruckert R, Brandt K, Bulanova E, Mirghomizadeh F, Paus R, Bulfone-Paus S: Dendritic cell–derived IL-15 controls the induction of CD8 T cell immune responses. Eur J Immunol 33:3493, 2003.
25. Chen YZ, Matsushita S, Nishimura Y: Response of a human T cell clone to a large panel of altered peptide ligands carrying single residue substitutions in an antigenic peptide: characterization and frequencies of TCR agonism and TCR antagonism with or without partial activation. J Immunol 157:3783, 1996.
26. Uemura Y, Senju S, Maenaka K, Iwai LK, Fujii S, Tabata H, Tsukamoto H, Hirata S, Chen YZ, Nishimura Y: Systematic analysis of the combinatorial nature of epitopes recognized by TCR leads to identification of mimicry epitopes for glutamic acid decarboxylase 65–specific TCRs. J Immunol 170:947, 2003.
27. Tabata H, Kanai T, Yoshizumi H, Nishiyama S, Fujimoto S, Matsuda I, Yasukawa M, Matsushita S, Nishimura Y: Characterization of self-glutamic acid decarboxylase 65–reactive $CD4^+$ T-cell clones established from Japanese patients with insulin-dependent diabetes mellitus. Hum Immunol 59:549, 1998.
28. Brunner T, Mogil RJ, LaFace D, Yoo NJ, Mahboubi A, Echeverri F, Martin SJ, Force WR, Lynch DH, Ware CF: Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. Nature 373:441, 1995.
29. Hugues S, Mougneau E, Ferlin W, Jeske D, Hofman P, Homann D, Beaudoin L, Schrike C, Von Herrath M, Lehuen A, Glaichenhaus N: Tolerance to islet antigens and prevention from diabetes induced by limited apoptosis of pancreatic beta cells. Immunity 16:169, 2002.
30. Chen YZ, Matsushita S, Nishimura Y: A single residue polymorphism at DR beta affects recognition of peptides by T cells. Hum Immunol 54:30, 1997.
31. Chen YZ, Lai ZF, Nishi K, Nishimura Y: Modulation of calcium responses by altered peptide ligands in a human T cell clone. Eur J Immunol 28:3929, 1998.

32. Lai ZF, Chen YZ, Nishimura Y, Nishi K: An amiloride-sensitive and voltage dependent Na^+ channel in an HLA-DR–restricted human T cell clone. J Immunol 165:83, 2000.
33. Marrack P, Mitchell T, Bender J, Hildeman D, Kedl R, Teague K, Kappler J: T-cell survival. Immunol Rev 165: 279, 1998.
34. Riley JL, Mao M, Kobayashi S, Biery M, Burchard J, Cavet G, Gregson BP, June CH, Linsley PS: Modulation of TCR-induced transcriptional profiles by ligation of CD28, ICOS, and CTLA-4 receptors. Proc Natl Acad Sci U S A 99:11790, 2002.
35. Brown JA, Dorfman DM, Ma FR, Sullivan EL, Munoz O, Wood CR, Greenfield EA, Freeman GJ: Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production. J Immunol 170: 1257, 2003.
36. Lechler R, Chai JG, Marelli-Berg F, Lombardi G: The contributions of T-cell anergy to peripheral T-cell tolerance. Immunology 103:262, 2001.
37. Wells AD, Walsh MC, Bluestone JA, Turka LA: Signaling through CD28 and CTLA-4 controls two distinct forms of T cell anergy. J Clin Invest 108:895, 2001.
38. Kanegane H, Tosato G: Activation of naive and memory T cells by interleukin-15. Blood 88:230, 1996.
39. LaSalle JM, Toneguzzo F, Saadeh M, Golan DE, Taber R, Hafler DA: T-cell presentation of antigen requires cell-to-cell contact for proliferation and anergy induction: differential MHC requirements for superantigen and autoantigen. J Immunol 151:649, 1993.
40. LaSalle JM, Tolentino PJ, Freeman GJ, Nadler LM, Hafler DA: Early signaling defects in human T cells anergized by T cell presentation of autoantigen. J Exp Med 176:177, 1992.
41. Lombardi G, Sidhu S, Batchelor R, Lechler R: Anergic T cells as suppressor cells *in vitro*. Science 264:1587, 1994.
42. Jenkins MK, Chen CA, Jung G, Mueller DL, Schwartz RH: Inhibition of antigen-specific proliferation of type 1 murine T cell clones after stimulation with immobilized anti-CD3 monoclonal antibody. J Immunol 144:16, 1990.
43. Koenen HJ, Fasse E, Joosten I: IL-15 and cognate antigen successfully expand de novo–induced human antigen-specific regulatory $CD4^+$ T cells that require antigenspecific activation for suppression. J Immunol 171:6431, 2003.
44. Horwitz DA, Zheng SG, Gray JD: The role of the combination of IL-2 and TGF beta or IL-10 in the generation and function of $CD4^+$ $CD25^+$ and $CD8^+$ regulatory T cell subsets. J Leukoc Biol 74:471, 2003.
45. Furtado GC, Curotto de Lafaille MA, Kutchukhidze N, Lafaille JJ: Interleukin 2 signaling is required for CD4(+) regulatory T cell function. J Exp Med 196:851, 2002.

Therapeutic effect of α-galactosylceramide-loaded dendritic cells genetically engineered to express SLC/CCL21 along with tumor antigen against peritoneally disseminated tumor cells

Hidetake Matsuyoshi,[1,2] Shinya Hirata,[1] Yoshihiro Yoshitake,[1] Yutaka Motomura,[1] Daiki Fukuma,[1] Akari Kurisaki,[1] Tetsuya Nakatsura,[1] Yasuharu Nishimura[1] and Satoru Senju[1,3]

[1]Department of Immunogenetics and [2]Department of Otolaryngology-Head and Neck Surgery, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan

(Received August 5, 2005/Revised September 23, 2005/Accepted September 29, 2005/Online publication December 8, 2005)

The close cooperation of both innate and acquired immunity is essential for the induction of truly effective antitumor immunity. We tested a strategy to enhance the cross-talk between NKT cells and conventional antigen-specific T cells with the use of αGalCer-loaded dendritic cells genetically engineered to express antigen plus chemokine, attracting both conventional T cells and NKT cells. DC genetically engineered to express a model antigen, OVA, along with SLC/CCL21 or monokine induced by IFN-γ/CXCL9, had been generated using a method based on *in vitro* differentiation of DC from mouse ES cells. The ES-DC were loaded with α-GalCer and transferred to mice bearing MO4, an OVA-expressing melanoma, and their capacity to evoke antitumor immunity was evaluated. *In vivo* transfer of either OVA-expressing ES-DC, stimulating OVA-reactive T cells, or α-GalCer-loaded non-transfectant ES-DC, stimulating NKT cells, elicited a significant but limited degree of protection against the i.p. disseminated MO4. A more potent antitumor effect was observed when α-GalCer was loaded to ES-DC expressing OVA before *in vivo* transfer, and the effect was abrogated by the administration of anti-CD8, anti-NK1.1 or anti-asialo GM1 antibody. α-GalCer-loaded double transfectant ES-DC expressing SLC along with OVA induced the most potent antitumor immunity. Thus, α-GalCer-loaded ES-DC expressing tumor-associated antigen along with SLC can stimulate multiple subsets of effector cells to induce a potent therapeutic effect against peritoneally disseminated tumor cells. The present study suggests a novel way to use α-GalCer in immunotherapy for peritoneally disseminated cancer. (*Cancer Sci* 2005; 96: 889–896)

A means to induce the close cooperation of both innate and acquired immunity would be necessary for the induction of efficient antitumor therapy. Recent studies have shown DC to be potent stimulators of both innate and acquired immunity. The *in vivo* transfer of DC presenting tumor-associated antigens has proven to be efficient in the priming of CTL specific to the antigens. α-GalCer presented by DC efficiently stimulates NKT cells,[1–4] a subset of T cells implicated in the innate immunity against infection and cancer.[5–7] In addition, NKT cells stimulated by the *in vivo* administration of α-GalCer secondarily stimulate conventional T cells.[8,9] It is thus presumed that the *in vivo* transfer of DC simultaneously loaded with tumor-associated antigens and α-GalCer may stimulate both tumor-reactive T cells and NKT cells, thus resulting in a potent antitumor immunity.

Chemokines mediate leukocyte adhesion and homing, and the concordant migration of specific leukocyte subsets induced by chemokines is pivotal for the development of proper immune responses. SLC/CCL21 attracts both T cells and DC to lymphoid tissues through its receptor CCR7, and the effect of SLC is essential for the priming of naive T cells in the initiation phase of the immune response. CXCR3 and its ligands, Mig/CXCL9 and IP-10/CXCL10, mediate the migration of effector/memory T cells and NK cells to the site of inflammation. In addition, a recent study revealed that these chemokines and their receptors also mediate the migration of some subpopulations of NKT cells.[10–12]

As a means for loading the tumor-associated antigens to DC, genetic modification to express antigenic proteins has several advantages. The expression of tumor antigens by DC circumvents the need for identifying specific CTL epitopes within the protein, and by that the antigens are continuously supplied for presentation as opposed to a single pulse of peptides or tumor cell lysates.[13] For the efficient gene transfer to DC, the use of virus-based vectors is required because DC are not easy to genetically modify. Considering the clinical application, however, there are several problems related to the use of virus vectors. These include the inefficiency of gene transfer, the instability of gene expression, and the potential risk accompanying the use of virus vectors. In addition, in many countries, legal restrictions have been placed on the use of virus vectors outside of carefully isolated laboratories.

We and others have established methods to generate dendritic cells *in vitro* from mouse ES cells.[14,15] ES-DC have the

[3]To whom correspondence should be addressed.
E-mail: senjusat@gpo.kumamoto-u.ac.jp

Abbreviations: Ab, antibody; BM-DC, bone marrow cell-derived dendritic cell; CBF1, (CBA × C57BL/6) F1; CTL, cytotoxic T lymphocyte; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; ES cell, embryonic stem cell; ES-DC, ES cell-derived dendritic cell; α-GalCer, α-galactosylceramide; GM-CSF, granulocyte macrophage colony-stimulating factor; HLA, human histocompatibility leukocyte antigen; IFN-γ, interferon-gamma; IL, interleukin; i.p., intraperitoneally; IP-10, IFN-γ-inducible 10 kDa protein; mAb, monoclonal antibody; Mig, monokine induced by IFN-γ; NK, natural killer; OVA, ovalbumin; s.c., subcutaneously; SLC, secondary lymphoid tissue chemokine.

doi: 10.1111/j.1349-7006.2005.00123.x

capacity to stimulate T cells, present antigen and migrate to lymphoid tissues upon *in vivo* administration, and these capacities of ES-DC are comparable to those of BM-DC. The genetic modification of ES-DC can be carried out without the use of virus vectors by introducing exogenous genes by electroporation into undifferentiated ES cells and the subsequent induction of their differentiation into ES-DC. We can generate multiple gene-transfectant ES-DC by the sequential transfection of ES cells with vectors bearing different selection markers.[16,17] In a previous study, we generated double-transfectant ES-DC expressing SLC or Mig along with a model tumor antigen.[16] Using these double-transfectant ES-DC, we demonstrated that the coexpression of SLC or Mig enhanced the capacity of *in vivo*-transferred ES-DC to activate antigen-specific CTL and to protect the recipient mice from a tumor cell challenge.

In the present study, we evaluated the capacity of α-GalCer-loaded ES-DC to stimulate NKT cells both *in vitro* and *in vivo*, in comparison to that of BM-DC. We next evaluated the antitumor effect of simultaneous stimulation of NKT cells and antigen-specific conventional T cells by the *in vivo* administration of α-GalCer-loaded ES-DC expressing a model tumor antigen, namely OVA. Furthermore, we addressed whether coexpression of SLC or Mig with the antigen by ES-DC could enhance the synergic antitumor effect of NKT cells and conventional T cells.

Materials and Methods

Mice

CBA and C57BL/6 mice were obtained from Clea Animal Co. (Tokyo, Japan) or Charles River (Hamamatsu, Japan) and kept under specific pathogen-free conditions. Male CBA and female C57BL/6 mice were mated to produce CBF1 mice and all *in vivo* experiments were carried out using the CBF1 mice. The animal experiments in this study were approved by the animal experiment committee of Kumamoto University (permission number A16-074).

Reagents

Recombinant mouse GM-CSF was purchased from PeproTech EC (London, UK) and α-GalCer was kindly provided by the Kirin Brewery Co. (Tokyo, Japan). Mouse IL-4 and IFN-γ ELISA kits were purchased from eBioscience (San Diego, CA, USA). Polyclonal rabbit anti-asialo GM1 Ab was purchased from Wako Chemicals (Tokyo, Japan).

Cell lines and preparation of DC

The ES cell line TT2, derived from CBF1 blastocysts,[18] was maintained as described previously.[19] MO4[20] was generated by the transfection of C57BL/6-derived melanoma B16 with the pAc-neo-OVA plasmid. The procedure for inducing the differentiation of ES cells into DC has been reproted previously.[15] ES-DC expressing OVA (ES-DC-OVA) and ES-DC expressing chemokine, SLC or Mig, along with OVA (ES-DC-OVA/SLC or ES-DC-OVA/Mig) were generated as reported previously.[16] ES-DC recovered after a 14-day culture in bacteriological Petri dishes were used for both *in vivo* and *in vitro* assays. To generate BM-DC, bone marrow cells were isolated from CBF1 mice and cultured in RPMI + 10% fetal calf serum + GM-CSF (5 ng/mL) for 7 days, according to the method reported by Lutz *et al.*[21]

Analysis of the activation of NKT cells by DC loaded with α-GalCer

Embryonic stem cell-derived dendritic cells or BM-DC were cultured in the presence of α-GalCer (100 ng/mL) or vehicle (0.00025% Polysorbate-20) alone for 22 h, washed twice, and cocultured with splenic T cells of syngeneic CBF1 mice (5×10^4 DC $+2.5 \times 10^6$ T cells/well in 24-well culture plates). Splenic T cells were isolated using nylon-wool columns, as described previously.[16] After 24 h or 5 days, the cells were recovered and analyzed on their cytotoxic activity by a 4-h ^{51}Cr-release assay using YAC-1 cells (1×10^4 cells/well) as targets in 96-well round-bottomed culture plates at the effector : target ratio indicated. The amount of IL-4 and IFN-γ in the culture supernatant was measured by ELISA. In the analysis of the stimulation of NKT cells *in vivo*, ES-DC or BM-DC loaded with either α-GalCer (100 ng/mL) or vehicle alone, as described above, were injected i.p. into syngeneic CBF1 mice (1×10^6 cells/mouse). After 24 h, the mice were killed and the cytotoxic activity of whole spleen cells against YAC-1 cells was analyzed, as described above.

Tumor challenge experiments

Indicated numbers of MO4 cells were injected s.c. into the shaved left flank region, or i.p. on day 0. On day 3, 1×10^5 genetically modified ES-DC preloaded with either α-GalCer or vehicle alone were transferred i.p. into the mice. The survival rate of the mice was monitored and, in s.c. challenge experiments, the tumor sizes were also determined biweekly in a blinded fashion. The tumor index was calculated as:

Tumor index (mm) = square root of (length × width).

In vivo depletion experiments

The mice were challenged i.p. with 1×10^5 MO4 cells on day 0 and they were injected i.p. with 1×10^5 ES-DC-OVA preloaded with α-GalCer on day 3. To deplete the specific types of cells, the mice were given a total of 14 i.p. injections (days −4, −1, 2, 5, 10, 13, 15, 19, 26, 33, 40, 47, 54 and 61) of mAb, ascites (0.1 mL/mouse/injection) from hybridoma-bearing nude mice, or polyclonal rabbit anti-asialo GM1 Ab (50 μg/mouse/injection). The mAbs used were rat antimouse CD4 (clone GK1.5), rat antimouse CD8 (clone 2.43) and mouse anti-NK1.1 (clone PK-136). Normal rat IgG (Sigma, St Louis, MO, USA) was used as a control (200 μg/mouse/injection).

Statistical analysis

Two-tailed Student's *t*-test was used to determine the statistical significance of differences in cytolytic activity and the tumor growth between the treatment groups. A value of $P < 0.05$ was considered to be significant. The Kaplan–Meier plot for survival was used to determine any significant differences in tumor challenge experiments, using the Breslow–Gehan–Wilcoxon test. In some experiments, the difference in the survival rate between treatment groups was assessed for significance using the χ^2-test. Statistical analyses were made using the StatView 5.0 software (Abacus Concepts, Calabasas, CA, USA).

doi: 10.1111/j.1349-7006.2005.00123.x

Results

Activation of NKT cells by ES-DC pulsed with α-GalCer

Mouse splenic DC and BM-DC loaded with α-GalCer have been reported to efficiently stimulate NKT cells, resulting in the rapid induction of NK cell-like cytolytic activity and the production of cytokines such as IL-4 and IFN-γ.[2,3] We examined whether ES-DC loaded with α-GalCer had the capacity to activate NKT cells, as naturally occurring DC do.

TT2 ES cell-derived non-transfectant ES-DC (ES-DC-TT2) or BM-DC were preincubated with α-GalCer, and then cocultured with splenic T cells isolated from syngeneic CBF1 mice. After 24 h, the cultured cells were recovered and their cytolytic activity against YAC-1 target cells was analyzed by a ^{51}Cr-release assay. The results shown in Fig. 1a indicate that a significant cytotoxity against YAC-1 cells was induced in the splenic T cell preparations by coculture with ES-DC loaded with α-GalCer, in comparison to the coculture with ES-DC loaded with vehicle alone. The cytotoxic activity induced by α-GalCer-loaded ES-DC-TT2 was comparable to that induced by α-GalCer-loaded BM-DC (Fig. 1a). As shown in Fig. 1b, IL-4 and IFN-γ were produced by splenic T cells cocultured with α-GalCer-loaded BM-DC or ES-DC, and a similar amount of the cytokines was produced in the culture with BM-DC and ES-DC preloaded with α-GalCer. If the coculture of T cells with α-GalCer-loaded ES-DC was extended to 5 days, the induced killing activity (Fig. 1c) and the amount of IL-4 and IFN-γ produced was increased in parallel (data not shown).

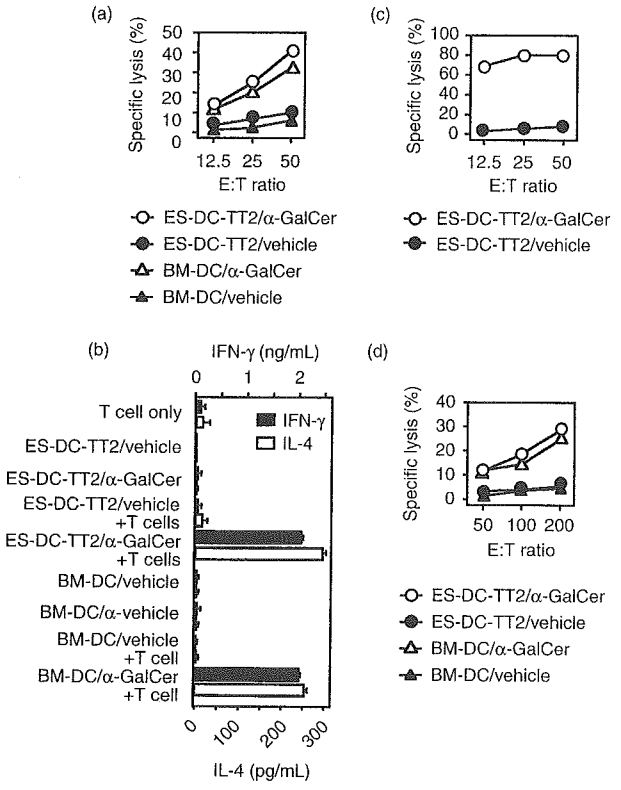

Fig. 1. Activation of NKT cells by the α-GalCer-loaded ES-DC. (a) ES-DC-TT2 or BM-DC were loaded with either α-GalCer (100 ng/mL) or vehicle (Polysorbate-20) alone for 22 h, washed extensively, and cocultured with splenic T cells of syngeneic CBF1 mice (5×10^4 DC + 2.5×10^6 T cells/well in 24-well culture plates). After 24 h of culture, the cells were recovered and the cytotoxic activity of the harvested cells against YAC-1 cells (1×10^4 cells) was analyzed using a 4-h Cr-release assay at the effector : target (E : T) ratios indicated. (b) Amounts of IL-4 and IFN-γ in the supernatant collected at the end of the 24-h coculture were quantified by ELISA. The results are expressed as the mean cytokine production of triplicate assays + SD. (c) The coculture was extended to 5 days and the killing activity of resultant cells was analyzed as in (a). (d) ES-DC or BM-DC were cultured in the presence of either α-GalCer (100 ng/mL) or vehicle alone for 18 h, washed, and injected i.p. into syngeneic CBF1 mice (1×10^6 cells/mouse). After 24 h, spleen cells were isolated from the mice and their cytotoxic activity against YAC-1 cells was analyzed as in (a). The results are expressed as the mean specific lysis of triplicate assays. The SD of triplicates were less than 2%.

We next analyzed the capacity of α-GalCer-loaded ES-DC to activate NKT cells *in vivo*. ES-DC-TT2 or BM-DC were preloaded with α-GalCer or vehicle alone in the same way as described above and i.p. injected into the syngeneic CBF1 mice. After 24 h, the mice were killed and the cytotoxic activity of whole spleen cells against YAC-1 cells was analyzed. As shown in Fig. 1d, a significant degree of cytotoxic activity was induced in the spleen cells by transfer of ES-DC loaded with α-GalCer, but it was not induced by the transfer of those loaded with vehicle alone. The capacity to evoke YAC-1 cell-killing activity of ES-DC and that of BM-DC was similar also *in vivo*. The activated NKT cells are known to activate the cytotoxic activity of NK cells.[22] It is therefore possible that the cytotoxic activity observed in these assays were mostly mediated by NK cells secondarily stimulated by NKT cells. Even so, these data collectively demonstrate that ES-DC had the capacity to present α-GalCer to activate NKT cells, and the capacity was similar to that of BM-DC both *in vitro* and *in vivo*.

Anti-tumor effect of α-GalCer-loaded ES-DC

We assessed whether the activation of NKT cells *in vivo* by α-GalCer-loaded ES-DC had any therapeutic effect against the tumor cells growing *in vivo*. MO4, originating from NK-sensitive B16 melanoma cells, were injected s.c. into the left flank region of mice and, 3 days later, the mice were treated with an i.p. injection of ES-DC-TT2 loaded with α-GalCer or vehicle alone. As shown in Fig. 2a, ES-DC loaded with α-GalCer did not show any therapeutic effect in this s.c. tumor model.

We next investigated the effect of α-GalCer-loaded ES-DC in the peritoneally disseminated tumor model. MO4 cells were injected i.p. into mice and, 3 days later, the mice were treated with an i.p. injection of ES-DC loaded with either α-GalCer or vehicle alone. As shown in Fig. 2b, which indicated the survival rate of the treated mice, the injection of ES-DC-TT2 loaded with α-GalCer elicited a significant ($P < 0.05$) but limited protective effect against the i.p. disseminated tumor cells.

Synergic therapeutic effect of α-GalCer-activated NKT cells and antigen-specific T cells against peritoneally disseminated tumor cells

In a previous study, we demonstrated that the *in vivo* transfer of ES-DC-OVA effectively primed OVA-specific CTL and induced protection against a subsequent challenge with s.c. injected MO4 cells expressing OVA.[16] We investigated

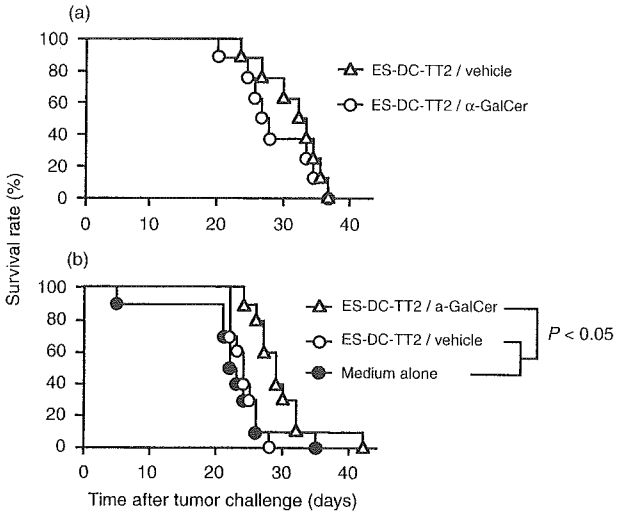

Fig. 2. Anti-tumor effect of *in vivo*-transferred α-GalCer-loaded ES-DC. The mice were (a) inoculated s.c. with MO4 cells (3×10^5 cells/mouse) to the left flank region or (b) inoculated i.p. with MO4 cells (1×10^5 cells/mouse). After 3 days, the mice were treated with i.p. injection of ES-DC-TT2 (1×10^5 cells/mouse) loaded with α-GalCer, vehicle alone or medium alone, and the mouse survival rate was monitored ($n = 10$ per group). In (b), the survival rate of the α-GalCer-loaded ES-DC-TT2-treated group was higher than that of the other two groups and the difference was statistically significant. Data are representative of four independent and reproducible experiments.

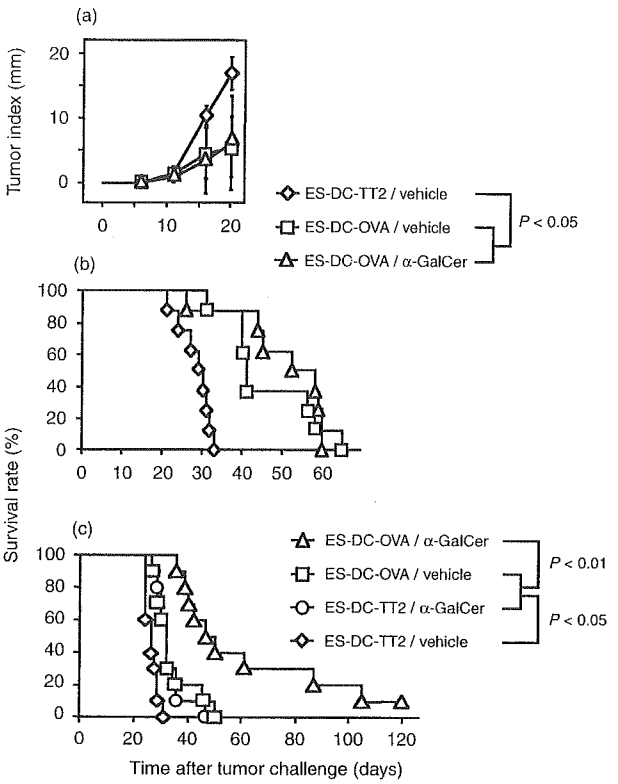

Fig. 3. Synergic effect of α-GalCer loading and the expression of tumor antigen on the protection against tumors induced by ES-DC. MO4 cells (3×10^5 cells/mouse) were injected s.c. into the left flank region of the mice and, 3 days later, the mice were treated with an i.p. injection of ES-DC-TT2 (1×10^5 cells/mouse) loaded with vehicle alone, ES-DC-TT2 loaded with α-GalCer, or ES-DC-OVA loaded with α-GalCer. After that, the tumor sizes were determined (a) and survival rate was monitored (b). Both the differences in tumor index (a) and in mouse survival rate (b) between the vehicle-loaded ES-DC-TT2-treated group and other two groups were statistically significant. (c) MO4 cells (1×10^5 cells/mouse) were injected i.p. into the mice and, 3 days later, the mice were treated with an i.p. injection of ES-DC-OVA or ES-DC-TT2 (1×10^5 cells/mouse) loaded with α-GalCer or vehicle alone. Thereafter, the mouse survival rate was monitored. The survival rate of the α-GalCer-loaded ES-DC-OVA-treated group was higher than that of the other groups and the difference was statistically significant. The survival rates of the vehicle-loaded ES-DC-OVA-treated group and α-GalCer-loaded ES-DC-TT2-treated group were higher than that of vehicle-loaded ES-DC-TT2-treated group and the difference was statistically significant. Each group included 10 mice. Data are representative of three independent and reproducible experiments.

whether the loading of α-GalCer to ES-DC-OVA before *in vivo* transfer would enhance the therapeutic effect against pre-established MO4 tumor. The mice were challenged s.c. with MO4 cells, and then 3 days later they were treated by i.p. injection of ES-DC-OVA preloaded with α-GalCer or vehicle alone. As shown in Fig. 3a,b, compared to the transfer of ES-DC-TT2, the transfer of ES-DC-OVA, loaded with either α-GalCer or vehicle alone, elicited a significant antitumor effect in this therapeutic model, as observed in the previously reported prevention (prophylactic) model.[16] However, the loading of α-GalCer to ES-DC-OVA did not improve the effect, based on either the tumor growth or the mouse survival time (Fig. 3a,b). These results suggest that the activation of NKT cells by α-GalCer loaded to ES-DC does not enhance the therapeutic effect of antigen-specific T cells against s.c. growing tumor cells.

We next investigated the effect of transfer of α-GalCer-loaded ES-DC-OVA against peritoneally disseminated tumor cells. Mice were i.p. inoculated with MO4 cells and 3 days later they were treated by an i.p. injection of ES-DC-OVA loaded with α-GalCer or vehicle alone, or ES-DC-TT2 loaded with vehicle alone. As shown in Fig. 3c, the therapeutic effect of the transfer of ES-DC-OVA loaded with vehicle alone was significant ($P < 0.05$) in comparison to the treatment with ES-DC-TT2 loaded with vehicle alone, but the effect was less marked than the effect observed in the s.c. growing tumor model (Fig. 3b). In contrast, the treatment with ES-DC-OVA loaded with α-GalCer elicited a potent effect to prolong the survival time of the mice. Given that the antitumor effect elicited by α-GalCer-loaded non-transfectant ES-DC was also limited (Figs 2b,3c), these results indicate that the NKT cells activated by α-GalCer presented by ES-DC and OVA-specific CTL primed by OVA antigen presented by the same ES-DC acted synergistically to protect the mice.

Subsets of effector cells contributing to the antitumor effect induced by α-GalCer-loaded ES-DC that expressed a model tumor antigen

To determine the effector cells exhibiting the observed antitumor effect induced by adoptive transfer of α-GalCer-loaded ES-DC expressing OVA, we carried out depletion experiments by injecting the mice with Abs specific to several subsets of effector cells during the tumor cell challenge and treatment with ES-DC. Figure 4a shows the

doi: 10.1111/j.1349-7006.2005.00123.x

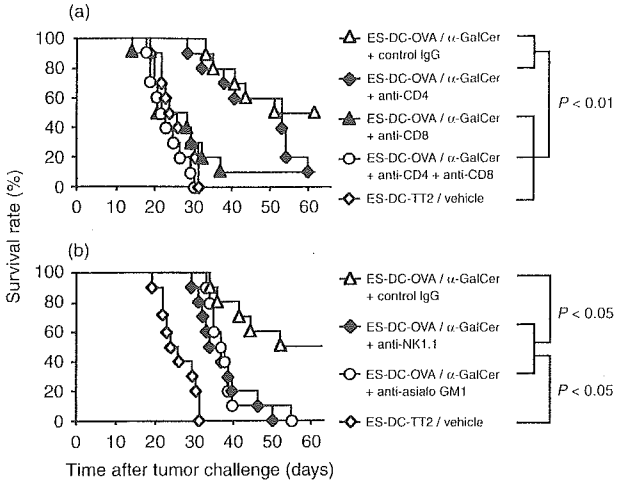

Fig. 4. Effector cells involved in the antitumor effect exerted by adoptive transfer of α-GalCer-loaded, antigen-expressing ES-DC. The mice were challenged i.p. with 1×10^5 MO4 cells on day 0 and injected i.p. with 1×10^5 ES-DC-OVA preloaded with α-GalCer on day 3. To deplete the specific types of cells, the mice were given i.p. injections of mAb or polyclonal rabbit anti-asialo GM1 Ab. The effect of the injection of anti-CD4, anti-CD8, or a combination of these two Abs is shown in (a). The effect of anti-NK1.1 or rabbit anti-asialo GM1 Ab is shown in (b). As a control, the survival of the mice treated with normal rat IgG is shown in both (a) and (b). Each group included 10 mice. In (a), the survival rates of α-GalCer-loaded ES-DC-OVA plus control IgG-treated group and α-GalCer-loaded ES-DC-OVA plus anti-CD4-treated group were higher than those of the other three groups and the difference was statistically significant. In (b), the survival rates of α-GalCer-loaded ES-DC-OVA plus anti-NK1.1 or anti-asialo GM1-treated groups and those of the other two groups were statistically different. The experiment was carried out once.

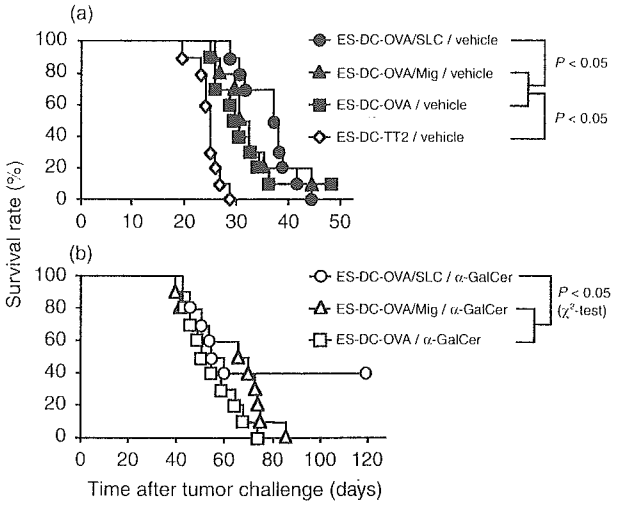

Fig. 5. Enhanced antitumor effect elicited by α-GalCer-loaded ES-DC expressing SLC along with OVA. (a) MO4 cells (1×10^5 cells/mouse) were injected i.p. into the mice and, 3 days later, the mice were treated with i.p. injection of ES-DC-TT2, ES-DC-OVA, ES-DC-OVA/Mig, or ES-DC-OVA/SLC (1×10^5 cells/mouse), all loaded with vehicle only. Thereafter, the survival rate of the mice was monitored. The differences in the survival rate between ES-DC-OVA/Mig-treated or ES-DC-OVA-treated group and the other two groups were statistically significant. (b) The mice were challenged with MO4 cells as in (a) and treated with either ES-DC-OVA, ES-DC-OVA/SLC or ES-DC-OVA/Mig, all loaded with α-GalCer. The frequency of mice from the ES-DC-OVA/SLC-treated group surviving for more than 100 days (four out of 10 mice) was significantly higher than that of the other two groups (0 out of 10 mice in each group), according to the χ^2-test. The experiment was carried out once.

effect of the injection of anti-CD4 or anti-CD8 mAbs or a combination of these two mAbs. The injection of anti-CD8 mAb almost totally abrogated the effect of the treatment with the ES-DC, thus suggesting that $CD8^+$ OVA-specific CTL played an important role in protecting the mice from the tumor. Compared to the effect of anti-CD8 mAb, the injection of anti-CD4 mAb had far less influence on the protective effect against the tumor, thus indicating the function of $CD4^+$ helper T cells to be not essential.

Figure 4b shows the effect of the injection of rabbit anti-asialo GM1 Ab, depleting NK cells, and also that of anti-NK1.1 mAb, depleting both NK and NKT cells. Treatment with either of these two kinds of Ab decreased the effect of α-GalCer-loaded ES-DC-OVA to a level similar to that elicited by vehicle-loaded ES-DC-OVA. These results indicate that NK cells played an essential role in the enhanced antitumor effect caused by the activation of NKT cells by α-GalCer.

Enhanced antitumor effect elicited by α-GalCer-loaded ES-DC expressing SLC along with OVA

We previously found that the coexpression of SLC or Mig, T cell-attracting chemokines that natural DC do not produce, along with OVA by ES-DC significantly enhanced their capacity to prime OVA-specific CTL and also to induce a protective immunity against s.c. injected MO4 cells.[16] A recent study revealed that these two chemokines induced chemotaxis not only of conventional T cells but also of some subpopulations of the NK cells and NKT cells.[10–12,23] We therefore examined whether the coexpression of such chemokine by ES-DC expressing OVA would also have an enhancing effect in protection against the i.p. growing MO4 cells.

We first assessed the effect of the expression of such chemokines by ES-DC without preloading with α-GalCer. We analyzed the capacity of ES-DC-OVA/SLC or ES-DC-OVA/Mig, ES-DC expressing OVA simultaneously with SLC or Mig, to induce protection against i.p. disseminated MO4 cells, comparing the capacity with that of ES-DC-OVA. The effect elicited by ES-DC-OVA/Mig was not higher than that elicited by ES-DC-OVA. Thus, the expression of Mig did not enhance the antitumor effect (Fig. 5a). However, expression of SLC by ES-DC did enhance the protective effect, although the effect of SLC in this i.p. tumor model was less evident than that observed in the s.c. tumor model reported previously.[16]

We next evaluated the effect of the expression of either SLC or Mig on the antitumor effect elicited by α-GalCer-loaded ES-DC-OVA. α-GalCer ES-DC-OVA/Mig and α-GalCer ES-DC-OVA exhibited a similar degree of protection, thus indicating that the coexpression of Mig by α-GalCer-loaded ES-DC-expressing OVA did not have any additive effect (Fig. 5b). In contrast, α-GalCer-loaded ES-DC-OVA/SLC exhibited a far more potent protective effect than α-GalCer ES-DC-OVA/Mig or α-GalCer ES-DC-OVA did. We observed that 40% of the mice treated with α-GalCer-loaded ES-DC-OVA/SLC completely rejected the tumor cells (Fig. 5b). These results suggest that the SLC produced by

ES-DC augmented the synergic effect of antigen-reactive CTL, α-GalCer-activated NKT cells, and probably NK cells.

As we reported previously,[16] coexpression of SLC along with OVA in ES-DC enhanced the capacity of ES-DC to prime OVA-specific CTL upon *in vivo* transfer. The data shown in the Fig. 5a also indicate that coexpression of SLC enhanced the capacity of ES-DC to induce antitumor immunity mediated by OVA-specific CTL in the absence of α-GalCer. To assess the effect of SLC produced by ES-DC on the activation of NKT or NK cells, we compared the capacity of α-GalCer-loaded ES-DC-OVA and α-GalCer-loaded ES-DC-OVA/SLC to stimulate NKT cells by an experiment similar to that shown in Fig. 1d. As a result, we observed that the capacity of α-GalCer-loaded ES-DC to induce YAC-1 cell-killing activity was not enhanced by expression of SLC (data not shown). Thus, effect of SLC produced by ES-DC to enhance the activation of NKT and NK cells was not detected at least by this short-term (24 h) assay. Based on these observations, it may be considered that the expression of SLC by ES-DC dominantly enhanced the activation of antigen-specific CTL rather than NKT or NK cells.

Discussion

In the present study, we evaluated the effect of loading α-GalCer to ES-DC expressing a model tumor antigen on their capacity to induce antitumor immunity. Upon loading with α-GalCer, ES-DC had a capacity comparable to that of BM-DC to stimulate NKT cells (Fig. 1). The *in vivo* administration α-GalCer-loaded non-transfectant ES-DC had some antitumor effect in an i.p. disseminated tumor model but not in an s.c. growing tumor model (Fig. 2). The difference in the effect of loading of α-GalCer to ES-DC in between the two models may be accounted for by the tissue distribution of NKT cells. NKT cells localize mainly in the liver, lung, spleen, bone marrow and peritoneal cavity.[6,11,24,25] In parallel with these observations, the loading of α-GalCer to ES-DC-OVA enhanced their antitumor effect against i.p. disseminated but not s.c. growing MO4 tumor cells (Fig. 3).

In a previous study, we observed that the protective effect against s.c. growing MO4 cells by transfer of ES-DC-OVA was almost totally abrogated by the depletion of either of CD4 or CD8 T cells.[16] In contrast, in the present study, the depletion of $CD8^+$ T cells but not $CD4^+$ T cells diminished the antitumor effect against i.p. MO4 cells elicited by α-GalCer-loaded ES-DC-OVA (Fig. 4a). These results indicate that CTL play a pivotal role in both conditions, and that $CD4^+$ T helper cells were not essential in the protective immunity against i.p. tumor cells on the occasion of simultaneous activation of NKT cells. The reason for the dispensability of $CD4^+$ T helper cells may be that NKT cells and NK cells, secondarily activated by NKT cells, provide help to OVA-specific CTL.[26] The data shown in Fig. 4b revealed that the depletion of NK cells decreased the effect of α-GalCer-loaded ES-DC-OVA to a degree similar to that elicited by vehicle-loaded ES-DC-OVA, indicating that NK cells played an essential role in the enhancement of the antitumor effect obtained by loading α-GalCer to ES-DC-OVA. Collectively, $CD8^+$ CTL, NKT cells and NK cells played essential roles in the antitumor effect obtained by α-GalCer to ES-DC expressing the antigen (Fig. 6). Presumably, the sequential stimulation of NKT cells and NK cells augmented the antitumor effect of OVA-specific CTL[9] and probably the interactions of effector cells were mediated by IFN-γ and IL-2.[22,27–31]

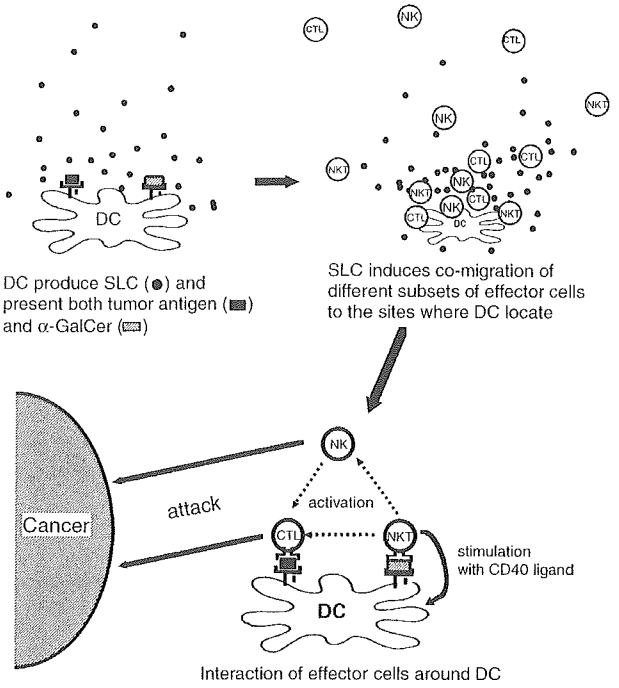

Fig. 6. A schematic depiction of the enhanced cross-talk of different subsets of effector cells induced by α-GalCer-loaded ES-DC expressing OVA plus SLC. SLC secreted by ES-DC induces the comigration of different subsets of effector cells, including NKT cells, NK cells and antigen-specific T cells, to the sites where the injected ES-DC are located. The effector cells of both innate and acquired immunity gathered around ES-DC, which present both α-GalCer and tumor antigen, thus closely interacting to develop a potent antitumor immunity.

The data shown in Fig. 5b indicate that the expression of SLC by ES-DC enhanced the antitumor effect induced by the transfer of α-GalCer-loaded ES-DC expressing OVA. SLC has been reported to attract not only conventional T cells and DC but also NKT cells.[10,23] SLC also induces chemotaxis of $CD56^{bright}$ $CD16^-$ NK cells and has a costimulatory effect on the proliferation of NK cells.[32] Thus, SLC probably induced the comigration of conventional T cells, NKT cells and NK cells to the sites where ES-DC were located, and, as a result, the close interaction of such multiple subsets of effector cells may have occurred (Fig. 6).

In the past decade, α-GalCer has been attracting attention as a novel immunostimulatory reagent for antitumor therapy. Based on the promising results of preclinical studies demonstrating antitumor effects of α-GalCer,[2,25,33] several phase I clinical studies on anticancer immunotherapy by the direct intravenous administration of α-GalCer or the administration of α-GalCer-loaded DC have been carried out.[34–37] Although the activation and expansion of NKT cells by the administration of α-GalCer has been observed, the results seemed to be unsatisfactory from the viewpoint of the clinical effect. The present study demonstrated that α-GalCer is useful for induction

doi: 10.1111/j.1349-7006.2005.00123.x

of immunity against peritoneally disseminated tumor cells, especially when it is loaded to DC genetically engineered to express tumor antigen. Although metastasis of melanoma to visceral organ sites is observed frequently in patients with advanced (stage IV) malignant melanoma, peritoneal dissemination of melanoma is very rare. Thus, we are planning another study with more clinical relevance, using models of cancer with a high tendency to peritoneal dissemination.

In recent years, a number of tumor-associated antigens have been identified. These antigens are potentially good targets for immunotherapy. To establish truly effective anticancer immunotherapy, a method for potently polarizing the immune system toward these antigens is essential. Antitumor immunotherapy with DC loaded with HLA-binding peptides derived from tumor antigens has been tested clinically in many institutions. In most cases, the DC are generated by culture of monocytes obtained from peripheral blood of the patients. To generate a sufficient number of DC for treatment, apheresis, a procedure that is sometimes invasive for patients with advanced stages of cancer, is necessary to obtain a sufficient number of monocytes as a source for DC. In addition, the culture to generate DC should be done separately for each patient and for each treatment, and thus the procedure used at present may be too labor-intensive and expensive to be applied broadly in a practical setting. Alternately, the source of ES-DC, ES cells, have the capacity to propagate infinitely. We would thus be able to use human ES cells as an infinite source of DC. In addition, we will be able to generate genetically engineered DC without the need to use virus vectors, as mentioned above. We may thus be able to generate multiple gene-transfectant ES-DC expressing tumor antigen plus immunostimulating molecules, which could be more potent in stimulating antitumor immunity than monocyte-derived DC are.

Regarding the future clinical application of ES-DC, we recently established a method for generating ES-DC from ES cells of a non-human primate, namely the cynomolgus monkey, and also for their genetic modification (unpublished data). We believe that this method would be applicable to human ES cells, although some modifications may be necessary. Considering the future clinical application of ES-DC technology, allogenicity (i.e. differences in the genetic background) between patients to be treated and ES cells as a source for DC may cause problems. However, it is expected that human ES cells sharing some HLA alleles with patients will be available for most cases. We recently found that antigen-expressing ES-DC potently primed antigen-specific CTL after the transfer to semiallogeneic mice sharing some MHC alleles with the ES-DC, and protected the recipient mice from subsequent challenge with tumor cells bearing the antigen.[38] CD1d is monomorphic and thus a CD1d-α-GalCer-complex on ES-DC can stimulate the NKT cells of any recipients. α-GalCer would thus be an ideal adjuvant to enhance the immune response toward the tumor antigens presented by ES-DC.

Acknowledgments

We thank Dr S. Aizawa for TT2, Drs N. Takakura and T. Suda for OP9, Dr K. Lock for MO4, Drs T. Nishimura, T. Koda and M. Bevan for a cDNA clone for OVA, and Kirin Brewery for α-GalCer. This work was supported in part by Grants-in-Aid 12213111, 14370115, 14570421 and 14657082 from the Ministry of Education, Science, Technology, Sports, and Culture, Japan, and a Research Grant for Intractable Diseases from Ministry of Health, Labor and Welfare, Japan, and grants from the Tokyo Biochemical Research Foundation and Uehara Memorial Foundation, and by funding from the Meiji Institute of Health Science.

References

1 Kitamura H, Iwakabe K, Yahata T *et al.* The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J Exp Med* 1999; **189**: 1121–8.
2 Toura I, Kawano T, Akutsu Y, Nakayama T, Ochiai T, Taniguchi M. Cutting edge: inhibition of experimental tumor metastasis by dendritic cells pulsed with α-galactosylceramide. *J Immunol* 1999; **163**: 2387–91.
3 Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged IFN-γ-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol* 2002; **3**: 867–74.
4 Bezbradica JS, Stanic AK, Matsuki N *et al.* Distinct roles of dendritic cells and B cells in Va14Ja18 natural T cell activation *in vivo*. *J Immunol* 2005; **174**: 4696–705.
5 Gonzalez-Aseguinolaza G, de Oliveira C, Tomaska M *et al.* α-Galactosylceramide-activated Vα 14 natural killer T cells mediate protection against murine malaria. *Proc Natl Acad Sci USA* 2000; **97**: 8461–6.
6 Kawamura T, Seki S, Takeda K *et al.* Protective effect of NK1.1$^+$ T cells as well as NK cells against intraperitoneal tumors in mice. *Cell Immunol* 1999; **193**: 219–25.
7 Johnson TR, Hong S, Van Kaer L, Koezuka Y, Graham BS. NK T cells contribute to expansion of CD8$^+$ T cells and amplification of antiviral immune responses to respiratory syncytial virus. *J Virol* 2002; **76**: 4294–303.
8 Nishimura T, Kitamura H, Iwakabe K *et al.* The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. *Int Immunol* 2000; **12**: 987–94.
9 Silk JD, Hermans IF, Gileadi U *et al.* Utilizing the adjuvant properties of CD1d-dependent NK T cells in T cell-mediated immunotherapy. *J Clin Invest* 2004; **114**: 1800–11.
10 Kim CH, Johnston B, Butcher EC. Trafficking machinery of NKT cells: shared and differential chemokine receptor expression among V alpha 24$^+$V beta 11$^+$ NKT cell subsets with distinct cytokine-producing capacity. *Blood* 2002; **100**: 11–16.
11 Liu Y, Poon RT, Hughes J, Feng X, Yu WC, Fan ST. Chemokine receptors support infiltration of lymphocyte subpopulations in human hepatocellular carcinoma. *Clin Immunol* 2005; **114**: 174–82.
12 Thomas SY, Hou R, Boyson JE *et al.* CD1d-restricted NKT cells express a chemokine receptor profile indicative of Th1-type inflammatory homing cells. *J Immunol* 2003; **171**: 2571–80.
13 Kaplan JM, Yu Q, Piraino ST *et al.* Induction of antitumor immunity with dendritic cells transduced with adenovirus vector-encoding endogenous tumor-associated antigens. *J Immunol* 1999; **163**: 699–707.
14 Fairchild PJ, Brook FA, Gardner RL *et al.* Directed differentiation of dendritic cells from mouse embryonic stem cells. *Curr Biol* 2000; **10**: 1515–18.
15 Senju S, Hirata S, Matsuyoshi H *et al.* Generation and genetic modification of dendritic cells derived from mouse embryonic stem cells. *Blood* 2003; **101**: 3501–8.
16 Matsuyoshi H, Senju S, Hirata S, Yoshitake Y, Uemura Y, Nishimura Y. Enhanced priming of antigen-specific CTLs *in vivo* by embryonic stem cell-derived dendritic cells expressing chemokine along with antigenic protein: application to antitumor vaccination. *J Immunol* 2004; **172**: 776–86.
17 Hirata S, Senju S, Matsuyoshi H, Fukuma D, Uemura Y, Nishimura Y. Prevention of experimental autoimmune encephalomyelitis by transfer of

embryonic stem cell-derived dendritic cells expressing myelin oligodendrocyte glycoprotein peptide along with TRAIL or programmed death-1 ligand. *J Immunol* 2005; **174**: 1888–97.
18 Yagi T, Tokunaga T, Furuta Y *et al.* A novel ES cell line, TT2, with high germline-differentiating potency. *Anal Biochem* 1993; **214**: 70–6.
19 Senju S, Iyama K, Kudo H, Aizawa S, Nishimura Y. Immunocytochemical analyses and targeted gene disruption of GTPBP1. *Mol Cell Biol* 2000; **20**: 6195–200.
20 Falo LD Jr, Kovacsovics-Bankowski M, Thompson K, Rock KL. Targeting antigen into the phagocytic pathway *in vivo* induces protective tumour immunity. *Nat Med* 1995; **1**: 649–53.
21 Lutz MB, Kukutsch N, Ogilvie AL *et al.* An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Meth* 1999; **223**: 77–92.
22 Eberl G, MacDonald HR. Selective induction of NK cell proliferation and cytotoxicity by activated NKT cells. *Eur J Immunol* 2000; **30**: 985–92.
23 Johnston B, Kim CH, Soler D, Emoto M, Butcher EC. Differential chemokine responses and homing patterns of murine TCR alpha beta NKT cell subsets. *J Immunol* 2003; **171**: 2960–9.
24 Takeda K, Seki S, Ogasawara K *et al.* Liver NK1.1$^+$ CD4$^+$ alpha beta T cells activated by IL-12 as a major effector in inhibition of experimental tumor metastasis. *J Immunol* 1996; **156**: 3366–73.
25 Nakagawa R, Motoki K, Ueno H *et al.* Treatment of hepatic metastasis of the colon26 adenocarcinoma with an α-galactosylceramide, KRN7000. *Cancer Res* 1998; **58**: 1202–7.
26 Stober D, Jomantaite I, Schirmbeck R, Reimann J. NKT cells provide help for dendritic cell-dependent priming of MHC class I-restricted CD8$^+$ T cells *in vivo*. *J Immunol* 2003; **170**: 2540–8.
27 Hayakawa Y, Takeda K, Yagita H *et al.* Critical contribution of IFN-γ and NK cells, but not perforin-mediated cytotoxicity, to anti-metastatic effect of α-galactosylceramide. *Eur J Immunol* 2001; **31**: 1720–7.
28 Carnaud C, Lee D, Donnars O *et al.* Cutting edge: Cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *J Immunol* 1999; **163**: 4647–50.
29 Smyth MJ, Crowe NY, Pellicci DG *et al.* Sequential production of interferon-γ by NK1.1$^+$ T cells and natural killer cells is essential for the antimetastatic effect of α-galactosylceramide. *Blood* 2002; **99**: 1259–66.
30 Chamoto K, Takeshima T, Kosaka A *et al.* NKT cells act as regulatory cells rather than killer cells during activation of NK cell-mediated cytotoxicity by α-galactosylceramide *in vivo*. *Immunol Lett* 2004; **95**: 5–11.
31 Wesley JD, Robbins SH, Sidobre S, Kronenberg M, Terrizzi S, Brossay L. Cutting edge: IFN-γ signaling to macrophages is required for optimal Vα14i NK T/NK cell cross-talk. *J Immunol* 2005; **174**: 3864–8.
32 Robertson MJ. Role of chemokines in the biology of natural killer cells. *J Leukoc Biol* 2002; **71**: 173–83.
33 Hayakawa Y, Rovero S, Forni G, Smyth MJ. Alpha-galactosylceramide (KRN7000) suppression of chemical- and oncogene-dependent carcinogenesis. *Proc Natl Acad Sci USA* 2003; **100**: 9464–9.
34 Nieda M, Okai M, Tazbirkova A *et al.* Therapeutic activation of Vα24$^+$Vβ11$^+$ NKT cells in human subjects results in highly coordinated secondary activation of acquired and innate immunity. *Blood* 2004; **103**: 383–9.
35 Giaccone G, Punt CJ, Ando Y *et al.* A phase I study of the natural killer T-cell ligand α-galactosylceramide (KRN7000) in patients with solid tumors. *Clin Cancer Res* 2002; **8**: 3702–9.
36 Ishikawa A, Motohashi S, Ishikawa E *et al.* A phase I study of α-galactosylceramide (KRN7000)-pulsed dendritic cells in patients with advanced and recurrent non-small cell lung cancer. *Clin Cancer Res* 2005; **11**: 1910–17.
37 Chang DH, Osman K, Connolly J *et al.* Sustained expansion of NKT cells and antigen-specific T cells after injection of α-galactosyl-ceramide loaded mature dendritic cells in cancer patients. *J Exp Med* 2005; **201**: 1503–17.
38 Fukuma D, Matsuyoshi H, Hirata S *et al.* Cancer prevention with semi-allogeneic ES cell-derived dendritic cells. *Biochem Biophys Res Commun* 2005; **335**: 5–13.

doi: 10.1111/j.1349-7006.2005.00123.x

Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Immunology Letters 100 (2005) 164–169
Immunology Letters
www.elsevier.com/locate/

Autoimmunity against neurofilament protein and its possible association with HLA-DRB1*1502 allele in glaucoma

Tsuyoshi Yano[a], Kazuhiro Yamada[a,*], Akira Kimura[a], Tetsuji Takeshita[a], Motozumi Minohara[b], Jun-ichi Kira[b], Satoru Senju[c], Yasuharu Nishimura[c], Hidenobu Tanihara[a]

[a] *Department of Ophthalmology and Visual Science, Kumamoto University Graduate School of Medical Sciences, 1-1-1 Honjo, Kumamoto 860-0811, Japan*
[b] *Department of Neurology, Neurological Institute, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashiku, Fukuoka 812-8582, Japan*
[c] *Division of Immunogenetics, Department of Neuroscience and Immunology, Kumamoto University Graduate School of Medical Sciences, 2-2-1 Honjo, Kumamoto 860-0811, Japan*

Received 11 February 2005; accepted 25 March 2005
Available online 28 April 2005

Abstract

Glaucoma is understood as a neurodegenerative disease and intraocular pressure has been regarded as the major risk factors for the optic nerve damages. However, recent studies suggested that several risk factors including autoimmunity are also shown to play important roles in glaucoma. To identify the retinal antigen in glaucoma, we used the serological analysis of recombinant cDNA expression libraries (SEREX) approach and quantified IgG antibodies directed against the identified antigens in an ELISA. We identified neurofilament protein and the prevalence of anti-bovine neurofilament light subunit (NF-L) autoantibodies in glaucomatous patients was significantly higher than in healthy controls and patients with other uveitic and optic nerve diseases ($P < 0.05$). In addition, our immunogenetic analysis showed a possible association between HLA-DRB1*1502 allele and the patients positive for anti-NF-L autoantibodies. It suggests that the HLA class II-linked gene may be involved in development of autoimmunity in patients with glaucoma.

Keywords: Glaucoma; Serological analysis of recombinant cDNA expression libraries (SEREX); Neurofilament protein; Autoantibodies; HLA-DRB1*1502 allele

1. Introduction

Glaucoma is classified as a neurodegenerative disease, in which the onset and progression of optic nerve damages results in visual field defects. Intraocular pressure (IOP) has been regarded as the major risk factor for the progression of optic nerve damage. However, recent clinical and experimental studies suggest that several risk factors such as ischemia, glutamate and genetic background also play important roles in the onset and progression of glaucoma [1,2]. These risk factors are likely associated with the occurrence of apoptotic cell death in retinal ganglion cells in glaucomatous eyes [3,4].

Recently, several investigators have suggested that autoimmunity directed against retinal proteins may be related to the development of glaucomatous optic neuropathy in glaucomatous patients. For example, Wax and his colleagues revealed that various autoantibodies reactive to retinal antigens such as rhodopsin, 27 kDa heat shock protein (hsp27), α–β crystalline, glycosaminoglycans, glutathione *S*-transferase (GST) were found in the sera of glaucomatous patients [5–8]. Also, a recent study using sera obtained from Japanese glaucomatous patients demonstrated the presence of anti-γ-enolase autoantibodies. Interestingly, vitreous

* Corresponding author. Tel.: +81 96 373 5247; fax: +81 96 373 5249.
E-mail address: yama@384.jp (K. Yamada).

0165-2478/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.imlet.2005.03.017

injection of the anti-γ-enolase autoantibodies resulted in retinal damage in rats [9]. On the other hand, Schwartz and her colleagues reported that T cell-mediated autoimmunity plays a crucial role in the progression of glaucomatous optic neuropathy in mice and rats, and suggested possibility of immunotherapy for neuroprotection against glaucomatous optic neuropathy [10,11]. Taken together, immunological investigation of the glaucomatous optic neuropathy may lead to the development of a novel concept for the treatment of glaucoma in addition to the presently used IOP-lowering treatments.

Herein, we present evidence that neurofilament protein may be a novel autoantigen that is involved in autoimmunity and leads to the development of glaucomatous optic neuropathy. Also, our immunological analysis revealed that there was a statistical association between the HLA class II allele and the presence of autoantibodies directed against neurofilament protein in patients which suggests that there is HLA-linked genetic control of autoimmune glaucoma.

2. Materials and methods

2.1. Patients

In this study, 65 patients with glaucoma were subjected to analysis. The mean age (±S.D.) in the patients was 61.3 ± 12.6 years old (range, 22–78 years old). The patients included 34 males and 31 females. In 30 of the 65 patients, the glaucoma was diagnosed as normal tension glaucoma (NTG) based upon (1) open iridocorneal angles without any peripheral anterior synechia, (2) no evidence of elevation in intraocular pressure (>21 mmHg), (3) the presence of glaucomatous (or glaucoma-like) changes in visual fields and optic nerve appearance and (4) exclusion of any other causes of optic neuropathy. The remaining 35 patients were diagnosed as having primary open angle glaucoma with glaucomatous optic neuropathy and had a history of high intraocular pressure (>21 mmHg). Additionally, in this study, 42 patients with Vogt–Koyanagi–Harada disease (VKH), 31 patients with Behçet's disease (BD) with ocular complications, 30 patients with the opticospinal form of multiple sclerosis (OS-MS) and 30 patients with the conventional form of multiple sclerosis (C-MS) were subjected to immunological analysis. Sixty-nine normal healthy volunteers were used as healthy controls in the present study. Peripheral venous blood samples were immediately subjected to serum separation and stored at −80 °C before use. All subjects were unrelated Japanese living in Japan and informed consents were obtained from all subjects prior to blood sampling. This study was done according to the tenets of the Declaration of Helsinki.

2.2. Immunoscreening

A rat retinal cDNA expression library was purchased from Strategene (La Jolla, CA, USA). The immunoscreening method used was a modification of our published methods [12,13]. Sera were diluted in Tris-buffered-saline (TBS). Recombinant phages at a concentration of 1×10^4/15 cm plate were amplified for 6 h at 42 °C, then transferred onto nitrocellulose filters Hybond-c extra (Amersham, Buckinghamshire, England), pretreated with isopropyl b-D-thiogalactoside (Wako, Osaka, Japan), and incubated for an additional 3 h at 37 °C to transfer the encoded proteins onto the filter membranes. Membranes were then removed from plate and blocked with 5% (w/v) skim milk/TBS. After washing with TBS containing 0.05% Tween 20 (TBS-T), membranes were incubated in 2 h at 27 °C. This was followed by incubation in horseradish peroxidase (HRP)-conjugated mouse anti-human IgG (Southern Biotechnology Associates Inc., Birmingham, AL) for 1 h at room temperature. The membranes were washed in TBS-T and TBS and incubated with ECL RPN 2106 (Amersham) for 1 min, and exposed to autoradiographic film to detect any antibody-reactive phage plaques. Positive recombinant clones were subcloned and retested for sera reactivity as described above. A total of 1.5×10^6 recombinant clones were screened, using sera from three glaucomatous patients.

2.3. Sequence analysis of identified cDNA clones

Immunoreactive phage clones were subjected to in vivo excision of pBluescript phagemid, using the ExAssist helper phage/SOLR strain system (Stratagene). Plasmid DNA was purified, using QIAGEN Plasmid Midi Kits (Tokyo, Japan) cDNA inserts were sequenced, using an ABI Prism (Perkin-Elmer, Norwalk, CT) automated DNA sequencer and for sequence alignments we used BLAST software on NCBI and Genone Net.

2.4. Enzyme-linked immunosorbent assay

Detection and tituration of antibodies reactive to purified bovine neurofilament protein (PROGEN, Heidelberg, Germany) were done, using a direct enzyme-linked immunosorbent assay (ELISA). Microtiter plates (96-well) (NUNC, Denmark) were coated with neurofilamemt protein in PBS (pH 7.4) for 10 h at 4 °C. The plates were then washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 5% skim milk/PBS for 2 h at room temperature. The plates were washed with PBS-T and incubated for 2 h at room temperature with serum samples diluted at 1:100 with 1% skim milk/PBS. The plates were washed in PBS-T and 100 μl of HRP-conjugated mouse anti-human IgG diluted at 1:2000 with 1% skim milk/PBS was added to each well and then followed by incubation at room temperature for 1 h. The plates were washed with PBS-T, and 100 μl solution of *o*-phenylenediamine (Sigma Fast: Sigma Chemical Co., St. Louis, MO) was added to each well. After 15 min, the reaction was stopped by adding 100 μl of 4 M H_2SO_4, and the optical density (OD) at 490 nm was determined, using a Model 550 microplate reader (Bio-Rad, Hercules, CA). The reactiv-

ities of autoantibodies are expressed as the relative value of the OD unit in which the OD value for the serum sample of patient TT was estimated to be 100 OD units.

2.5. HLA-DRB1 typing

Extraction of genomic DNAs was performed by sodium dodecyl sulphate and proteinase C treated peripheral blood cells from 55 patients with glaucoma and 57 healthy controls. HLA-DRB1 genes were amplified by polymerase chain reaction (PCR) method and typed by the PCR-restriction fragment length polymorphism (RFLP) method as described elsewhere [14].

3. Results

3.1. Identification of immunoreactive cDNA clones

With a serum sample obtained from a patient with NTG (a 58-year-old man), 4.0×10^5 phage plaques in the rat retinal cDNA library were immunoscreened. Seven positive immunoreactive clones were identified. Their partial sequences of inserted DNAs and subsequent computer analysis revealed that they were derived from two different sequences. The deduced amino acid sequences of six clones were identical to rat neurofilament protein and another clone encoded for the Wiskott-Aldrich syndrome proteins family verprolin homologous protein3 (WAVE3). Thus, our immunoscreening studies revealed the presence of anti-rat neurofilament protein IgG antibodies in a patient with NTG.

3.2. Comparison of the prevalence of anti-bovine neurofilament IgG in sera from patients with glaucoma, other diseases and healthy controls

To determine if the autoimmunity directed against the neurofilament protein is associated with glaucomatous optic nerve damage, serum samples obtained from 65 glaucomatous patients, 42 patients with VKH, 31 patients with BD, 60 patients with MS and 69 healthy controls were tested for their reactivity to neurofilament protein. Because purified human neurofilament protein was not available for the present studies, bovine neurofilament protein (68-kD light subunit) was purchased from PROGEN (Heidelberg, Germany) and was used for the following experiments to determine the levels of antibody titers in ELISA in sera from the various patients and normal volunteers. Computer analysis of the deduced amino acid sequences demonstrated by the homology of the 68-kD light subunit of neurofilament (NF-L) between bovine and human, human and rat, rat and bovine was 78–81%.

Fig. 1 shows scatter grams of individual serum titers of the anti-NF-L IgG. The results were expressed as the relative value of OD unit. To determine the cut-off level for positivity of anti-NF-L in the 69 healthy controls, titer levels were measured. The mean (±S.D.) titer level of the anti-NF-L IgG was 7.5 ± 8.7. Thus, we regarded the mean value plus two times the standard deviation, 7.5 + 8.7 × 2 = 24.9, as the cut-off level for this ELISA analysis. The prevalence of anti-NF-L IgG in patients and healthy controls is shown in Table 1. Our ELISA experiments showed that the measured titer levels of anti-NF-L IgG were higher than the cut-off level, found in 12 (18.5%) of the 65 glaucomatous patients and in four (5.8%) of the 69 normal controls ($P = 0.022$, Fisher's exact test). The difference in the percentage of autoantibody-positive subjects was statistically significant between these two groups.

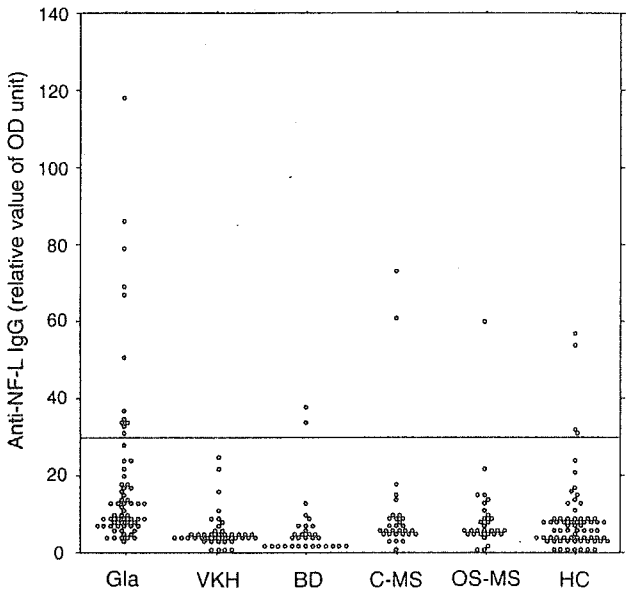

Fig. 1. Distribution of the titer of anti-bovine NF-L IgG antibodies in patients with glaucoma (Gla), Vogt–Koyanagi–Harada disease (VKH), Behçet's disease (BD), opticospinal form of multiple sclerosis (OS-MS), conventional form of multiple sclerosis (C-MS) and healthy controls (HC). Data were expressed as the relative value of optical density (OD) unit in which the OD value for the serum sample of patient TT was estimated to be 100 OD units. To determine the cut-off level for the positivity of anti-NF-L IgG, the levels were measured in 69 healthy controls. The mean (±S.D.) titer level of the anti-NF-L IgG was 7.5 ± 8.7. Thus, we regarded the mean value plus two times the standard deviation (24.9), as the cut-off level for this ELISA analysis (indicated by the line).

Table 1
Prevalence of anti-NF-L IgG evaluated by ELISA in sera from patients with glaucoma, disease controls and healthy controls

Diagnosis	Anti-NF-L IgG positive donors	Frequency (%)
Glaucoma	12/65	18.5*
VKH	0/42	0.0
BD	2/31	6.5
OS-MS	1/30	3.3
C-MS	2/30	6.7
HC	4/69	5.8*

VKH, Vogt–Koyanagi–Harada disease; BD, Behçet's disease; OS-MS, opticospinal form of multiple sclerosis; C-MS, conventional form of multiple sclerosis; HC, healthy control.

* Statistically significant difference in frequency of anti-NF-L IgG autoantibodies between HC and glaucom groups ($P = 0.022$, Fisher's exact test).

Table 2
Allele frequency of HLA-DRB1 in patients with glaucoma and normal controls

Allele	Glaucoma ($n=55$)		Control subject ($n=57$)		P-value
	n	AF	n	AF	
DRB1*0101	8	0.145	9	0.158	NS
DRB1*0301	1	0.018	1	0.018	NS
DRB1*0401	1	0.018	1	0.018	NS
DRB1*0403	2	0.036	2	0.035	NS
DRB1*0405	16	0.291	12	0.211	NS
DRB1*0406	4	0.073	5	0.088	NS
DRB1*0410	2	0.036	2	0.035	NS
DRB1*0802	4	0.073	3	0.053	NS
DRB1*0803	10	0.182	12	0.211	NS
DRB1*0901	17	0.301	15	0.263	NS
DRB1*1101	1	0.018	3	0.053	NS
DRB1*1201	1	0.018	4	0.07	NS
DRB1*1301	1	0.018	1	0.018	NS
DRB1*1302	8	0.145	9	0.158	NS
DRB1*1401	2	0.036	0	0	NS
DRB1*1405	4	0.073	3	0.053	NS
DRB1*1406	3	0.055	0	0	NS
DRB1*1501	8	0.145	8	0.14	NS
DRB1*1502	16	0.291	14	0.246	NS
DRB1*1602	1	0.018	1	0.018	NS

AF: allele frequency; NS: not significant; Chi-square test.

We measured antibody titer levels of anti-NF-L IgG in serum samples obtained from patients with various ocular diseases. ELISA studies showed that the titer levels higher than the cut-off value were found in none (0%) of the 42 patients with VKH, two (6.5%) of the 31 patients with BD, one (3.3%) of the 30 patients with OS-MS and two (6.7%) of the 30 patients with C-MS. As shown in Table 1, there were no statistically significant differences in the prevalence of the titer levels of anti-NF-L IgG that were higher than the cut-off level between the normal controls and each group. The age distribution is not significantly different between anti-NF-L IgG positive and negative donors in each group of patients as well as healthy controls (data not shown).

3.3. HLA-DRB1 allele frequency

There were no statistically significant differences in the allele frequency of HLA-DRB1 between normal controls and the combined glaucomatous patient group (Table 2). We divided the glaucomatous patients into two groups based upon their anti NF-L IgG titer levels. In the anti NF-L IgG positive group, which was composed of patients with anti NF-L IgG titer levels higher than the cut-off level, HLA-DRB1*1502 allele was observed in seven (58.3%) of the 12 patients. On the other hand, in the anti-NF-L IgG negative group, which was composed of patients with anti-NF-L IgG titer levels equal to or below the cut-off level, the allele was only found in seven (16.3%) of the 43 patients. The differences in prevalence of anti NF-L IgG were statistically significant between these two groups ($P<0.05$, Chi-square test) (Table 3).

Table 3
Difference between anti-NF-L IgG positive patients and negative patients in frequency of DRB1 alleles

Allele	Anti-NF-L IgG positive patients ($n=12$)		Anti-NF-L IgG negative patients ($n=43$)		P-Value
	n	Frequency	n	Frequency	
DRB1*0101	1	0.083	5	0.116	NS
DRB1*0301	0	0.000	1	0.023	NS
DRB1*0401	0	0.000	1	0.023	NS
DRB1*0403	0	0.000	2	0.047	NS
DRB1*0405	4	0.333	10	0.233	NS
DRB1*0406	0	0.000	4	0.093	NS
DRB1*0410	0	0.000	2	0.047	NS
DRB1*0802	0	0.000	4	0.093	NS
DRB1*0803	2	0.167	6	0.14	NS
DRB1*0901	2	0.167	11	0.256	NS
DRB1*1101	1	0.083	0	0	NS
DRB1*1201	0	0.000	1	0.023	NS
DRB1*1301	1	0.083	0	0	NS
DRB1*1302	2	0.167	6	0.14	NS
DRB1*1401	0	0.000	2	0.047	NS
DRB1*1405	1	0.083	3	0.07	NS
DRB1*1406	2	0.167	1	0.023	NS
DRB1*1501	1	0.083	7	0.163	NS
DRB1*1502	6	0.500	9	0.21	<0.05
DRB1*1602	0	0.000	1	0.023	NS

NS: not significant; Chi-square test.

4. Discussion

Recent studies have revealed that glaucomatous optic neuropathy may be caused by many risk factors such as ischemia, genetic background, glutamate and immune response in addition to intraocular pressure [1]. Among these, involvement of the immune response in the pathogenesis of glaucomatous optic neuropathy has been the focus of investigations. Several investigators have reported the presence of autoantibodies in glaucomatous patients [5–8,15,16]. Also, recent studies have suggested that T cell-mediated immune response might play a crucial role in the progression of glaucomatous optic nerve damages in animal experimental models [10,11,17,18]. These findings have shed light on new aspects for understanding of the pathogenesis and treatment of glaucomatous optic neuropathy. Based on these findings, we conducted a series of experiments in the present study in an attempt to further elucidate a role for involvement of autoimmunity and the immunogenetic background, in glaucomatous optic nerve damage.

In previous studies, Western blot analysis was used for the identification of autoantibodies in serum samples obtained from glaucomatous patients [5–8,16]. These methods are useful for identification of autoantibodies directed against antigens that have a large amount of expression. However, this method has a potential risk in that it can exclude the detection of important antigens if the autoantigens are barely expressed. For this reason it is possible that autoantigens associated with the progression of glaucomatous optic neuropathy

may escape detection of a Western blot analysis. Thus, in order to better understand the immunological mechanisms of glaucomatous optic neuropathy, more sensitive and reliable technique needs to be applied. In the present study, we applied SEREX method to the identification of glaucoma-associated autoantigens. This molecular biological method enabled us to detect autoantigens regardless of whether the expression was very low, and to easily accomplish subsequent molecular cloning of genes that encode for the autoantigen.

With the aid of the SEREX method in our previous studies, we have been able to identify disease-associated autoantigens in patients with VKH and cancers [12,19–21]. In VKH, T helper 1 type T cells-mediated cellular immunity plays a dominant role in the pathogenesis [22,23]. In cases of glaucoma, some investigators have suggested the involvement of T cell-mediated cellular immunity play a role in the neuroprotection against glaucomatous optic neuropathy whereas T helper 2 type T cells-mediated humoral immunity should be regarded as the main cause for the production of autoantibodies [11,17]. Thus, presently, our understanding on immunological mechanisms associated with glaucomatous optic neuropathy is far from satisfactory. Therefore, we considered the use of SEREX to be suitable for our aims of elucidating the mechanism of autoimmunity in glaucoma.

In the present study, we identified neurofilament proteins as the candidate target autoantigens associated with glaucoma. Neurofilament proteins are known to be major intermediate filaments that are present in the nervous system and construct the axonal cytoskeleton. So far, three types of subunits for neurofilament have been identified; the 68-kD light subunit (NF-L), the 160-kD medium subunit (NF-M) and the 200-kD heavy subunit (NF-H). NF-L constructs the backbone of neurofilament while NF-M and NF-H are localized on the periphery of NF-L. Targeted disruption of the NF-L gene results in reduction of radial growth of neuronal axons [24]. It has been reported that abnormal accumulation of neurofilament is the pathological hallmark of some neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Lewy-body-type dementia and Parkinson's disease [25–27]. The presence of autoantibodies directed against NF proteins has been reported in patients with progressive MS [28,29]. From the viewpoint of glaucoma, it has been reported that expression of NF proteins in retinal ganglion cells reduced in glaucomatous patients [30,31].

In our study, use of the SEREX method and a serum sample of a patient with normal tension glaucoma, allowed us to identify neurofilament protein as a candidate autoantigen associated with glaucomatous optic neuropathy. Our results showed a statistically significant difference between the prevalence of anti-NF-L IgG in normal controls and glaucomatous patients, but not between normal controls and other diseases such as VKH, BD and MS. In glaucoma, retinal ganglion cells and their axons specifically deteriorate whereas it is the uveal tissues that are mainly involved in VKH. However, in BD and MS as well as glaucoma, optic nerve damage dose occur. Thus, our positive results that showed the prevalence of a high titer of anti-NF-L IgG in glaucomatous patients might suggest that this immune response may be associated with a certain sub-group of glaucomatous patients. One of the most important points that need to be addressed concerns what mechanisms segregate this sub-group of glaucomatous patients with regards to the immunological reaction against NF-L. This question may be partially answered by our additional results for the typing of HLA class II alleles in glaucomatous patients.

The association between glaucoma and HLA class II allele has been previously reported [32,33]. In the present study, we document the frequency of HLA-DRB1 allele in Japanese glaucomatous patients for the first time. A statistically significant difference was revealed in the allele frequency of HLA-DRB1*1502 between the anti-NF-L IgG positive and negative groups in glaucomatous patients. Our finding suggests that specific HLA class II alleles or genes in with a strong linkage with disequilibrium with HLA-DRB1*1502 may play an important role in the production of autoantibodies in glaucomatous patients. The presence of autoantibodies reactive to retinal and/or optic nerve antigens may result from prolonged exposure of the immune system to autoantigens caused by glaucomatous optic nerve damage. However, we cannot exclude another possibility that the autoantibody-associated immune response may deteriorate (or inhibit) the progression of glaucomatous optic neuropathy. Taken into consideration together, we can hypothesize that the immunogenetic background associated with HLA class II alleles may control susceptibility to autoimmunity. Our hypothesis may shed new light for understanding the presence of autoantibodies in glaucomatous patients. Further studies are necessary to elucidate the relationships between HLA class II alleles and the immunological abnormality in glaucoma.

In conclusion, we have identified neurofilament proteins as a novel autoantigens associated with glaucomatous optic nerve damage. This autoimmunity was found in some but not all glaucomatous patients, and was not found in patients with other ocular diseases. HLA-DRB1*1502 allele was also significantly associated with high titer of anti-NF-L IgG in glaucomatous patients, suggesting the importance of HLA class II-linked genes in the development of autoimmunity in glaucoma.

References

[1] Vorwerk CK, Gorla MS, Dreyer EB. Surv Ophthalmol 1999;43:S142–50.
[2] Osborne NN, Ugarte M, Chao M, Chidlow G, Bae JH, Wood JP, et al. Surv Ophthalmol 1999;43:S102–28.
[3] Kerrigan LA, Zack DJ, Quigley HA, Smith SD, Pease ME. Arch Ophthalmol 1997;115:1031–5.
[4] Quigley HA, Nickells RW, Kerrigan LA, Pease ME, Thibault DJ, Zack DJ. Invest Ophthalmol Vis Sci 1995;36:774–86.
[5] Romano C, Barrett DA, Li Z, Pestronk A, Wax MB. Invest Ophthalmol Vis Sci 1995;36:1968–75.
[6] Tezel G, Seigel GM, Wax MB. Invest Ophthalmol Vis Sci 1998;39:2277–87.

[7] Tezel G, Edward DP, Wax MB. Arch Ophthalmol 1999;117:917–24.
[8] Yang J, Tezel G, Patil RV, Romano C, Wax MB. Invest Ophthalmol Vis Sci 2001;42:1273–6.
[9] Maruyama I, Maeda T, Okisaka S, Mizukawa A, Nakazawa M, Ohguro H. Jpn J Ophthalmol 2002;46:1–12.
[10] Fisher J, Levkovitch-Verbin H, Schori H, Yoles E, Butovsky O, Kaye JF, et al. J Neurosci 2001;21:136–42.
[11] Schori H, Kipnis J, Yoles E, WoldeMussie E, Ruiz G, Wheeler LA, et al. Proc Natl Acad Sci USA 2001;98:3398–403.
[12] Yamada K, Senju S, Nakatsura T, Murata Y, Ishihara M, Nakamura S, et al. Biochem Biophys Res Commun 2001;280:1169–76.
[13] Nakatsura T, Senju S, Ito M, Nishimura Y, Itoh K. Eur J Immunol 2002;32:826–36.
[14] Shindo Y, Inoko H, Yamamoto T, Ohno S. Br J Ophthalmol 1994;78:223–6.
[15] Wax MB, Tezel G, Kawase K, Kitazawa Y. Ophthalmology 2001;108:296–302.
[16] Maruyama I, Ohguro H, Ikeda Y. Invest Ophthalmol Vis Sci 2000;41:1657–65.
[17] Schwartz M. Surv Ophthalmol 2001;45:S256–60 (discussion S273–6).
[18] Bakalash S, Kipnis J, Yoles E, Schwartz M. Invest Ophthalmol Vis Sci 2002;43:2648–53.
[19] Yamada K, Senju S, Shinohara T, Nakatsura T, Murata Y, Ishihara M, et al. Immunol Lett 2001;78:161–8.
[20] Nakatsura T, Senju S, Yamada K, Jotsuka T, Ogawa M, Nishimura Y. Biochem Biophys Res Commun 2001;281:936–44.
[21] Monji M, Senju S, Nakatsura T, Yamada K, Sawatsubashi M, Inokuchi A, et al. Biochem Biophys Res Commun 2002;294:734–41.
[22] Yamaki K, Gocho K, Hayakawa K, Kondo I, Sakuragi S. J Immunol 2000;165:7323–9.
[23] Gocho K, Kondo I, Yamaki K. Invest Ophthalmol Vis Sci 2001;42:2004–9.
[24] Zhu Q, Couillard-Despres S, Julien JP. Exp Neurol 1997;148:299–316.
[25] Julien JP. Curr Opin Neurobiol 1999;9:554–60.
[26] Al-Chalabi A, Andersen PM, Nilsson P, Chioza B, Andersson JL, Russ C, et al. Hum Mol Genet 1999;8:157–64.
[27] Goldman JE, Yen SH, Chiu FC, Peress NS. Science 1983;221:1082–4.
[28] Silber E, Semra YK, Gregson NA, Sharief MK. Neurology 2002;58:1372–81.
[29] Eikelenboom MJ, Petzold A, Lazeron RH, Silber E, Sharief M, Thompson EJ, et al. Neurology 2003;60:219–23.
[30] Naskar R, Wissing M, Thanos S. Invest Ophthalmol Vis Sci 2002;43:2962–8.
[31] Vickers JC, Hof PR, Schumer RA, Wang RF, Podos SM, Morrison JH. Aust NZ J Ophthalmol 1997;25:239–43.
[32] Gil-Carrasco F, Vargas-Alarcon G, Zuniga J, Tinajero-Castaneda O, Hernandez-Martinez B, Hernandez-Pacheco G, et al. Am J Ophthalmol 1999;128:297–300.
[33] Ferreri G, D'Andrea A, Castagna I, Rechichi C, Pettinato G, D'Andrea D. Acta Ophthalmol Scand Suppl 1998:17–9.

nature genetics

A functional variant in *FCRL3*, encoding Fc receptor-like 3, is associated with rheumatoid arthritis and several autoimmunities

Yuta Kochi[1,2], Ryo Yamada[1], Akari Suzuki[1], John B Harley[3], Senji Shirasawa[4], Tetsuji Sawada[2], Sang-Cheol Bae[5], Shinya Tokuhiro[1], Xiaotian Chang[1], Akihiro Sekine[6], Atsushi Takahashi[7], Tatsuhiko Tsunoda[7], Yozo Ohnishi[8], Kenneth M Kaufman[3], Changsoo Paul Kang[9], Changwon Kang[9], Shigeru Otsubo[10], Wako Yumura[11], Akio Mimori[4], Takao Koike[12], Yusuke Nakamura[10,13], Takehiko Sasazuki[4] & Kazuhiko Yamamoto[1,2]

Rheumatoid arthritis is a common autoimmune disease with a complex genetic etiology. Here we identify a SNP in the promoter region of *FCRL3*, a member of the Fc receptor-like family, that is associated with susceptibility to rheumatoid arthritis (odds ratio = 2.15, $P = 0.00000085$). This polymorphism alters the binding affinity of nuclear factor-κB and regulates *FCRL3* expression. We observed high *FCRL3* expression on B cells and augmented autoantibody production in individuals with the disease-susceptible genotype. We also found associations between the SNP and susceptibility to autoimmune thyroid disease and systemic lupus erythematosus. *FCRL3* may therefore have a pivotal role in autoimmunity.

Rheumatoid arthritis is one of the most common autoimmune diseases and is characterized by inflammation of synovial tissue and joint destruction. Although the disease is believed to result from a combination of genetic and environmental factors, its complete etiology has not yet been clarified[1]. Specific haplotypes of human leukocyte antigen (HLA)-DRB1, usually referred to as shared-epitope sequences[2], have been repeatedly reported to confer susceptibility to rheumatoid arthritis[3,4]; other genetic components are also involved[5]. This combination of HLA haplotypes and non-HLA genes accounting for disease susceptibility is also observed for other autoimmune diseases[6–8]. In autoimmune thyroid disease (AITD), for instance, the *HLA-DR3* haplotype is associated with disease risk, as is a functional haplotype of a non-HLA gene, *CTLA4*, that has recently been associated with AITD susceptibility[9].

Identification of non-HLA genes associated with rheumatoid arthritis susceptibility and other autoimmunities seems difficult, because of the low relative risk of disease resulting from these non-HLA genes compared with the strong relative risk from disease-associated HLA haplotypes. In a search for non-HLA determinants of disease susceptibility, whole-genome studies have been done for both human autoimmune diseases and experimental animal models. These studies have identified nonrandom clustering of susceptibility loci for clinically distinct diseases[8,10]. The overlap of susceptibility loci for multiple autoimmunities suggests that common susceptibility genes exist in those regions. Intense studies of loci-clustering regions identified genes commonly associated with multiple autoimmune diseases, such as *CTLA4* on 2q33 (ref. 9), *SLC22A4* and *SLC22A5* on 5q31 (ref. 11) and *PTPN22* on 1p13 (ref. 12).

Cytoband 1q21–23 is one of the regions implicated in susceptibility to multiple autoimmune diseases. The Fcγ receptor (FcγR) II/III genes are located at 1q23, and a new family of genes, Fc receptor-like genes (FCRLs, also known as FcRHs[13,14], IRTAs[15,16] or SPAPs[17]), clusters nearby at 1q21 (**Fig. 1a**). FCRLs have high structural homology with classical FcγRs, although their ligands and function are not yet known. These receptors are good candidates for involvement in autoimmunity, as they are believed to be involved in the pathogenesis of rheumatoid arthritis and other autoimmune diseases[18]. Region 1q23 is a candidate locus for susceptibility to systemic lupus erythematosus

[1]Laboratory for Rheumatic Diseases, SNP Research Center, RIKEN, Yokohama 230-0045, Japan. [2]Department of Allergy and Rheumatology, Graduate School of Medicine, the University of Tokyo, Tokyo 113-0033, Japan. [3]University of Oklahoma; US Department of Veterans Affairs; and Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104, USA. [4]International Medical Center of Japan, Tokyo 162-8655, Japan. [5]Department of Internal Medicine, Division of Rheumatology, the Hospital for Rheumatic Diseases, Hanyang University, Seoul 133-792, Republic of Korea. Laboratories for [6]Genotyping, [7]Medical Informatics and [8]SNP Analysis, SNP Research Center, RIKEN, Yokohama 230-0045, Japan. [9]Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Republic of Korea. [10]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, the University of Tokyo, Tokyo 108-8639, Japan. [11]Department of Medicine, Kidney Center, Tokyo Women's Medical University, Tokyo 162-8666, Japan. [12]Department of Medicine II, Hokkaido University School of Medicine, Sapporo 060-8638, Japan. [13]Research Group for Personalized Medicine, SNP Research Center, RIKEN, Yokohama 230-0045, Japan. Correspondence should be addressed to R.Y. (ryamada@src.riken.go.jp).

Published online 17 April 2005; doi:10.1038/ng1540

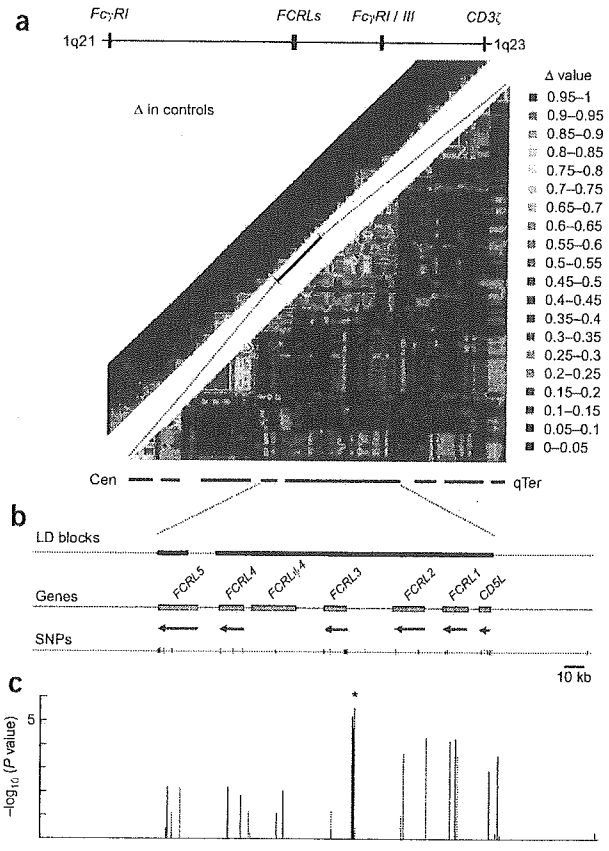

Figure 1 LD and association of the FCRL gene cluster. (**a**) Pairwise LD between SNPs, as measured by Δ in 658 controls. The 16-Mb region in 1q21–23 (upper left) and the 2-Mb region around the FCRL gene cluster (lower right) were evaluated. (**b**) Location of LD blocks, genes and 41 SNPs in the FCRL gene cluster. (**c**) Case-control association test with 41 SNPs in the FCRL gene cluster using 830 affected individuals and 658 controls. *Peak association.

(SLE), and variants in the classical FcγR II/III genes partially account for disease susceptibility[6,19]. Region 1q21 is a candidate locus for susceptibility to psoriasis (*PSORS4*; refs. 7,20) and multiple sclerosis[21]. The mouse homologous region to human 1q21, on chromosome 3, also contains susceptibility loci for multiple autoimmune disease models[8], including collagen-induced arthritis (*Cia5*, also called *Mcia2* (ref. 22); *Eae3* (ref. 23); *Tmevd2* (ref. 24); *Idd10*; and *Idd17* (ref. 25)). Although 1q21–23 is a good candidate region for containing rheumatoid arthritis–susceptibility genes, the association of classical FcγRs with disease susceptibility remains controversial[26,27]. Here we focused on the 1q21–23 region to identify rheumatoid arthritis–associated genes in Japanese subjects using linkage disequilibrium (LD) mapping.

RESULTS

Case-control study by SNP-based LD mapping at 1q21–23

To evaluate the extent of association, we analyzed LD with SNPs distributed in a 16-Mb region on 1q21–23, including the FCRL gene cluster and the classical FcγRs (**Fig. 1a**). We genotyped 658 control subjects for 742 SNPs from the JSNP database and selected 491 SNPs with allele frequency >0.1, successful genotyping rate >0.95 and $P > 0.01$ with Hardy-Weinberg equilibrium testing for evaluation of LD. We calculated the pairwise LD index Δ (ref. 28) for each pair of SNPs, identifying 110 LD blocks[11] at a threshold of $\Delta > 0.5$ (**Fig. 1a**).

For association testing, we examined the Japanese set of 830 cases and 658 controls used for LD block evaluation. We initially genotyped 94 rheumatoid arthritis cases for 491 SNPs and compared their allele frequencies with those of 658 control subjects. We identified nine SNPs that had allele frequencies differing by more than 0.1 between 658 controls and 94 cases with $P < 0.01$. We genotyped the remaining cases for these nine SNPs and tested their allele frequencies for case-control association. We identified the smallest P value between an intronic SNP in the gene *FCRL3* and rheumatoid arthritis (fcrl3_6, $P = 1.8 \times 10^{-5}$; association was statistically significant in both rheumatoid arthritis subgroups (94 and 736 individuals)). This SNP was located in a LD block containing four of the five FCRL genes; the fifth was in the adjacent block. We therefore evaluated the origin of this association in these two LD blocks (**Fig. 1b**), although our results do not exclude the presence of variants associated with rheumatoid arthritis or other autoimmune diseases in other LD blocks at 1q21–23.

In addition to the 25 SNPs of the 491 that we used for LD block evaluation, we identified 16 additional SNPs in exons and 5′ and 3′ flanking regions of five FCRL genes and one pseudogene (*FCRLψ4*) by searching the public database and sequencing genomic DNA from Japanese individuals with rheumatoid arthritis. We genotyped these 16 SNPs in the identical case and control samples (830 cases, 658 controls) to increase the density of variants in the targeted region. We observed a peak of association in a short segment consisting of four SNPs in *FCRL3* ($P < 1.0 \times 10^{-4}$; **Fig. 1c** and **Supplementary Table 1** online): fcrl3_3, fcrl3_4, fcrl3_5 and fcrl3_6, located at nt −169, −110, +358 (5′ untranslated region of exon 2) and +1381 (intron 3; 204 bp and 859 bp from the 3′ and 5′ ends of the flanking exons) relative to the transcription initiation site, respectively.

We observed the smallest P value without correction in recessive-trait genotype comparison of fcrl3_3 in *FCRL3* ($P = 8.5 \times 10^{-7}$; odds ratio = 2.15; 95% confidence interval = 1.58–2.93; **Table 1**). This

Table 1 Case-control analysis of *FCRL3*

			Allele 1 frequency		Genotype 11 versus 12 + 22		
SNP	Location	Allele (1/2)	Affected individuals	Controls	OR (95% c.i.)	χ^2	P
fcrl3_3	−169	C/T	0.42	0.35	2.15 (1.58–2.93)	24.3	0.00000085
fcrl3_4	−110	A/G	0.25	0.18	3.01 (1.71–5.29)	16.1	0.000060
fcrl3_5	Exon 2	C/G	0.42	0.35	2.05 (1.51–2.78)	21.6	0.0000033
fcrl3_6	Intron 3	A/G	0.42	0.34	2.02 (1.49–2.75)	20.8	0.0000052

SNPs with $P < 0.0001$ in allele frequency comparison test are shown. c.i., confidence interval; OR, odds ratio.