RNA and protein levels. RNA was extracted from B-EBV and SV40-fibroblasts in TRIzol (Invitrogen), and cDNA was prepared using reverse transcriptase (SuperScript II; Invitrogen) for RT-PCR, according to the manufacturer's instructions. Proteins for Western blotting were extracted from B-EBV and SV40-fibroblasts, and Western blots were probed with rabbit antibodies against IRAK-4 (Tularik) and GAPDH (Santa Cruz Biotechnology, Inc.).

TLR agonists. TLR agonists and cytokines were used at the following final concentrations, unless otherwise indicated: synthetic triacylated lipopeptide (PAM_3CSK_4, agonist of TLR1/2; Invivogen), 100 ng/ml; synthetic diacylated lipopeptide (PAM_2CSK_4, agonist of TLR2/6; Invivogen), 100 ng/ml; poly(I:C) (a synthetic analogue of dsRNA, polyinosine-polycytidylic acid, and nonspecific TLR3 agonist; Invivogen), 25 μg/ml; LPS (Re 595 from *Salmonella minnesota*, agonist of TLR-4; Sigma-Aldrich), 100 ng/ml; flagellin (TLR5 agonist; Invivogen), 1 μg/ml; 3M-13 (TLR7 agonist) and 3M-2 (TLR8 agonist; both provided by 3M Pharmaceuticals), 3 μg/ml each; R-848, resiquimod hydrochloride (TLR7 and TLR8 agonist; provided by PharmaTech), 3 μg/ml; and unmethylated CpG DNA CpG-C (C274; 5′-TCGTCGAACGTTCGAGATGAT-3′; TLR9 agonist; provided by R. Coffman and F. Barrat, Dynavax Technologies, Berkeley, CA), 3 μg/ml. Polymyxin B was used at 10 μg/ml (Sigma-Aldrich).

B-EBV and SV40-fibroblast activation. We suspended 10^6 B-EBV cells per well in RPMI 1640 (Invitrogen) supplemented with 10% FCS (Invitrogen) and activated them by incubation with 3M-13, 3M-2, R-848, and 10^{-7} M PMA plus 10^{-5} M ionomycin (Sigma-Aldrich) for 24 h. 10^5 SV40-fibroblast cells per well were seeded in DMEM (Invitrogen) supplemented with 10% FCS in 24-well plates. Cells were activated with 20 ng/ml TNF-α (R&D Systems), 10 ng/ml IL-1β (R&D Systems) and 10^{-7} M PMA plus 10^{-5} M ionomycin the next day. The supernatants were harvested after 24 h of activation.

Cytokine measurement. ELISA determinations of TNF-α, IL-6, and IL-10 in cell culture supernatants were performed with a kit (PeliPair reagent set; Sanquin), according to the manufacturer's instructions. Optical density was determined by an automated ELISA reader (MR5000; Thermolab Systems). We used a fluorescence-based assay (a human cytokine 25-plex antibody bead kit) that can detect 25 cytokines (LHC0009; Biosource International) for the simultaneous determination of multiple cytokines. Fluorescence was measured with a 100 IS system (Luminex Corporation). The assay and analysis were performed according to the manufacturer's instructions.

Cell purification and activation. Blood samples from healthy controls or patients were collected into heparin-containing tubes, and PBMCs and granulocytes were separated by Ficoll-gradient centrifugation. The patients were of different ages when the experiments were performed, ranging from 7 to 32 yr old. For granulocyte isolation, erythrocytes were lysed and washed twice in PBS. More than 95% of the granulocytes purified on Ficoll were $CD15^+$. We did not purify granulocytes by flow cytometry, as the surface expression and TLR-induced shedding of L-selectin (CD62L) were not better detected (unpublished data).The PBMC preparation was enriched in T cells, B cells, monocytes, and NK cells by magnetic bead isolation using anti-CD3, -CD19, -CD14, and -CD56 microbeads (Miltenyi Biotec), according to the manufacturer's instructions. Purified T cells were labeled with anti-CD3–FITC (BD Biosciences), B cells with anti-CD19–PE (BD Biosciences), monocytes with anti-CD14–FITC (BD Biosciences), and NK cells with anti-CD3–FITC/anti-CD56–PE (BD Biosciences) antibodies, and sorting was performed on a flow cytometer (FACSVantage; BD Biosciences). The isolated cells were cultured in RPMI 1640 supplemented with 10% FCS, with immediate TLR agonist stimulation. We added 100 U/ml IL-2 to cultures of purified T cells. Purified B cells were suspended in RPMI 1640 supplemented with 10% FCS at a density of 10^6 cells/ml. Cells were stimulated with TLR agonists together with 100 U/ml IL-4 for 3 d.

Analysis of selectin (CD62L) shedding on granulocytes. Granulocytes were isolated as described in the previous section, activated with TLR agonists, stained with anti-CD62L–FITC (BD Biosciences) antibody, and analyzed by flow cytometry, as previously described (10).

Ex vivo analysis of PDCs and MDCs. PBMCs were suspended at a final density of 2×10^6 cells/ml in RPMI supplemented with 10% FCS. They were incubated at 37°C, under an atmosphere containing 5% CO_2, and stimulated with TLR agonists. 10 μg/ml brefeldin A was added after 1 h of activation. After 3.5 h of activation, cells were washed and stained with anti-Lin1–FITC (BD Biosciences), anti-HLADR–PerCP (BD Biosciences), and anti-CD123–PE-Cy7 (e-Bioscience) antibodies. For intracellular staining, PBMCs were permeabilized with the Cytofix/Cytoperm kit (BD Biosciences), according to the manufacturer's instructions. Anti–TNF-α–allophycocyanin (BD Biosciences) and anti–MIP-1β–PE (BD Biosciences) antibodies were used to assess the response of MDCs and PDCs to TLR agonists. PBMCs were also incubated with the respective isotype controls, and cells were acquired on a three-laser flow cytometer (LSR system; BD Biosciences). MDCs were defined as $Lin\text{-}1^-$, $HLA\text{-}DR^+$, and $CD123^{low}$, and PDCs were defined as $Lin\text{-}1^-$, $HLA\text{-}DR^+$, and $CD123^{high}$. For analysis, the quadrant for each individual tested was set such that 98% of PBMCs incubated with the respective isotype controls were negative for nonspecific staining.

MDDCs. MDDCs were prepared as previously described (80). In brief, PBMCs were suspended in RPMI 1640 supplemented with 10% FCS, plated in cell culture flasks, and incubated for 1 h. Monocytes attached to the bottom of the culture flask and nonadherent cells were removed with medium. Monocytes were then cultured in RPMI 1640 supplemented with 10% FCS, 25 ng/ml GM-CSF, and 100 U/ml IL-4. GM-CSF and IL-4 were added to the medium every other day to maintain their initial concentrations. On day 7 or 8, some of the MDDCs were stained for CD1a and CD14. Living cells and cell debris were distinguished by forward/side scatter. More than 95% of living cells were $CD1a^+$, and no $CD14^+$ cells were detected. On day 7 or 8, MDDCs were suspended in RPMI 1640 supplemented with 10% FCS at a density of 2×10^5cells/ml, and supernatants were collected after 24 h of activation. The up-regulation of surface markers was assessed by collecting MDDCs and staining them with anti-CD1a–PE (BD Biosciences), anti-CD40–FITC (BD Biosciences), anti-CD80–FITC (BD Biosciences), and anti-CD86–FITC (BD Biosciences) antibodies.

Vaccination schedules of patients. Patients were immunized against diphtheria and tetanus in accordance with international recommendations. Nine patients received multiple injections of glycan antigens (nonconjugated ["Pneumo23"] and conjugated ["Prevenar"] antipneumococcal vaccine), and their specific antibody titers were subsequently monitored in detail.

Online supplemental material. Fig. S1 demonstrates a deletion of the *IRAK4* locus on one allele in P2 and the presence of both *IRAK4* loci in P7. Fig. S2 shows the detailed results for each of the 11 cytokines for which a response to TLR agonists in healthy controls could be detected by multiplex assay. Table S1 shows blood leukocyte subsets in IRAK-4–deficient patients. Table S2 highlights T cell proliferation, Ig levels, and humoral responses to recall antigens and to glycans in IRAK-4–deficient patients. Table S3 offers the serology of patients to common viruses. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20070628/DC1.

We thank all members of the laboratory for helpful discussions, and Catherine Bidalled, Martine Courat, and Tony Leclerc for secretarial and technical assistance. We would particularly like to thank the patients and their families, whose trust, support, and cooperation were essential for collection of the data used in this study.

H. von Bernuth was supported by grants from the Deutsche Forschungsgemeinschaft (VO 995/1-1 and VO 995/1-2), from the European Ph.D. program of the San Raffaele Institute, and from the program "Legs Poix" of the Parisian Universities. L. Marodi was supported by a grant from the Hungarian Research Fund (OTKA 49017), and A. Puel was supported by a grant from the European Union (QLK2-CT-2002-00846). The Laboratory of Human Genetics of

Infectious Diseases is supported by the March of Dimes, the BNP Paribas Foundation, the Dana Foundation, and the Schlumberger Foundation. J.L Casanova is an International Scholar of the Howard Hughes Medical Institute.

The authors have no conflicting financial interests.

Submitted: 28 March 2007
Accepted: 21 August 2007

REFERENCES

1. Picard, C., A. Puel, M. Bonnet, C.L. Ku, J. Bustamante, K. Yang, C. Soudais, S. Dupuis, J. Feinberg, C. Fieschi, et al. 2003. Pyogenic bacterial infections in humans with IRAK-4 deficiency. *Science*. 299:2076–2079.
2. Kuhns, D.B., D.A. Long Priel, and J.I. Gallin. 1997. Endotoxin and IL-1 hyporesponsiveness in a patient with recurrent bacterial infections. *J. Immunol.* 158:3959–3964.
3. Haraguchi, S., N.K. Day, R.P. Nelson Jr., P. Emmanuel, J.E. Duplantier, C.S. Christodoulou, and R.A. Good. 1998. Interleukin 12 deficiency associated with recurrent infections. *Proc. Natl. Acad. Sci. USA*. 95:13125–13129.
4. Medvedev, A.E., A. Lentschat, D.B. Kuhns, J.C. Blanco, C. Salkowski, S. Zhang, M. Arditi, J.I. Gallin, and S.N. Vogel. 2003. Distinct mutations in IRAK-4 confer hyporesponsiveness to lipopolysaccharide and interleukin 1 in a patient with recurrent bacterial infections. *J. Exp. Med.* 198:521–531.
5. Currie, A.J., D.J. Davidson, G.S. Reid, S. Bharya, K.L. MacDonald, R.S. Devon, and D.P. Speert. 2004. Primary immunodeficiency to pneumococcal infection due to a defect in Toll-like receptor signaling. *J. Pediatr.* 144:512–518.
6. Chapel, H., A. Puel, H. von Bernuth, C. Picard, and J.L. Casanova. 2005. *Shigella sonnei* meningitis due to interleukin-1 receptor-associated kinase-4 deficiency: first association with a primary immune deficiency. *Clin. Infect. Dis.* 40:1227–1231.
7. Takada, H., H. Yoshikawa, M. Imaizumi, T. Kitamura, J. Takeyama, S. Kumaki, A. Nomura, and T. Hara. 2006. Delayed separation of the umbilical cord in two siblings with interleukin-1 receptor-associated kinase 4 deficiency: rapid screening by flow cytometer. *J. Pediatr.* 148: 546–548.
8. Cardenes, M., H. von Bernuth, A. Garcia-Saavedra, E. Santiago, A. Puel, C.L. Ku, J.F. Emile, C. Picard, J.L. Casanova, E. Colino, et al. 2006. Autosomal recessive interleukin-1 receptor-associated kinase 4 deficiency in fourth-degree relatives. *J. Pediatr.* 148:549–551.
9. Davidson, D.J., A.J. Currie, D.M. Bowdish, K.L. Brown, C.M. Rosenberger, R.C. Ma, J. Bylund, P.A. Campsall, A. Puel, C. Picard, et al. 2006. IRAK-4 mutation (Q293X): rapid detection and characterization of defective post-transcriptional TLR/IL-1R responses in human myeloid and non-myeloid cells. *J. Immunol.* 177:8202–8211.
10. von Bernuth, H., C.L. Ku, C. Rodriguez-Gallego, S. Zhang, B.Z. Garty, L. Marodi, H. Chapel, M. Chrabieh, R.L. Miller, C. Picard, et al. 2006. A fast procedure for the detection of defects in Toll-like receptor signaling. *Pediatrics*. 118:2498–2503.
11. McDonald, D.R., D. Brown, F.A. Bonilla, and R.S. Geha. 2006. Interleukin receptor-associated kinase-4 deficiency impairs Toll-like receptor-dependent innate antiviral immune responses. *J. Allergy Clin. Immunol.* 118:1357–1362.
12. Ku, C.L., C. Picard, M. Erdos, A. Jeurissen, J. Bustamante, A. Puel, H. von Bernuth, O. Filipe-Santos, H.H. Chang, T. Lawrence, et al. 2007. IRAK4 and NEMO mutations in otherwise healthy children with recurrent invasive pneumococcal disease. *J. Med. Genet.* 44:16–23.
13. Yang, K., A. Puel, S. Zhang, C. Eidenschenk, C.L. Ku, A. Casrouge, C. Picard, H. von Bernuth, B. Senechal, S. Plancoulaine, et al. 2005. Human TLR-7-, -8-, and -9-mediated induction of IFN-alpha/beta and -lambda is IRAK-4 dependent and redundant for protective immunity to viruses. *Immunity*. 23:465–478.
14. Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda, and S. Akira. 2003. Role of adaptor TRIF in the MyD88-independent Toll-like receptor signaling pathway. *Science*. 301:640–643.
15. Hoebe, K., X. Du, P. Georgel, E. Janssen, K. Tabeta, S.O. Kim, J. Goode, P. Lin, N. Mann, S. Mudd, et al. 2003. Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature*. 424:743–748.
16. Kawai, T., O. Takeuchi, T. Fujita, J. Inoue, P.F. Muhlradt, S. Sato, K. Hoshino, and S. Akira. 2001. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J. Immunol.* 167:5887–5894.
17. Oshiumi, H., M. Matsumoto, K. Funami, T. Akazawa, and T. Seya. 2003. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat. Immunol.* 4:161–167.
18. Hayashi, F., T.K. Means, and A.D. Luster. 2003. Toll-like receptors stimulate human neutrophil function. *Blood*. 102:2660–2669.
19. Kadowaki, N., S. Ho, S. Antonenko, R.W. Malefyt, R.A. Kastelein, F. Bazan, and Y.J. Liu. 2001. Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens. *J. Exp. Med.* 194:863–869.
20. Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdorfer, T. Giese, S. Endres, and G. Hartmann. 2002. Quantitative expression of Toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J. Immunol.* 168:4531–4537.
21. Ito, T., R. Amakawa, T. Kaisho, H. Hemmi, K. Tajima, K. Uchira, Y. Ozaki, H. Tomizawa, S. Akira, and S. Fukuhara. 2002. Interferon α and interleukin 12 are induced differentially by Toll-like receptor 7 ligands in human blood dendritic cell subsets. *J. Exp. Med.* 195: 1507–1512.
22. Krug, A., S. Rothenfusser, V. Hornung, B. Jahrsdorfer, S. Blackwell, Z.K. Ballas, S. Endres, A.M. Krieg, and G. Hartmann. 2001. Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. *Eur. J. Immunol.* 31:2154–2163.
23. Hasan, U., C. Chaffois, C. Gaillard, V. Saulnier, E. Merck, S. Tancredi, C. Guiet, F. Briere, J. Vlach, S. Lebecque, et al. 2005. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. *J. Immunol.* 174:2942–2950.
24. Gorden, K.B., K.S. Gorski, S.J. Gibson, R.M. Kedl, W.C. Kieper, X. Qiu, M.A. Tomai, S.S. Alkan, and J.P. Vasilakos. 2005. Synthetic TLR agonists reveal functional differences between human TLR7 and TLR8. *J. Immunol.* 174:1259–1268.
25. Renn, C.N., D.J. Sanchez, M.T. Ochoa, A.J. Legaspi, C.K. Oh, P.T. Liu, S.R. Krutzik, P.A. Sieling, G. Cheng, and R.L. Modlin. 2006. TLR activation of Langerhans cell-like dendritic cells triggers an antiviral immune response. *J. Immunol.* 177:298–305.
26. Nagase, H., S. Okugawa, Y. Ota, M. Yamaguchi, H. Tomizawa, K. Matsushima, K. Ohta, K. Yamamoto, and K. Hirai. 2003. Expression and function of Toll-like receptors in eosinophils: activation by Toll-like receptor 7 ligand. *J. Immunol.* 171:3977–3982.
27. Dasari, P., I.C. Nicholson, G. Hodge, G.W. Dandie, and H. Zola. 2005. Expression of Toll-like receptors on B lymphocytes. *Cell. Immunol.* 236:140–145.
28. Caron, G., D. Duluc, I. Fremaux, P. Jeannin, C. David, H. Gascan, and Y. Delneste. 2005. Direct stimulation of human T cells via TLR5 and TLR7/8: flagellin and R-848 up-regulate proliferation and IFN-gamma production by memory CD4+ T cells. *J. Immunol.* 175: 1551–1557.
29. Tabiasco, J., E. Devevre, N. Rufer, B. Salaun, J.C. Cerottini, D. Speiser, and P. Romero. 2006. Human effector CD8+ T lymphocytes express TLR3 as a functional coreceptor. *J. Immunol.* 177:8708–8713.
30. Wesch, D., S. Beetz, H.H. Oberg, M. Marget, K. Krengel, and D. Kabelitz. 2006. Direct costimulatory effect of TLR3 ligand poly(I:C) on human gamma delta T lymphocytes. *J. Immunol.* 176:1348–1354.
31. Suzuki, N., S. Suzuki, G.S. Duncan, D.G. Millar, T. Wada, C. Mirtsos, H. Takada, A. Wakeham, A. Itie, S. Li, et al. 2002. Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. *Nature*. 416:750–756.
32. Suzuki, N., S. Suzuki, D.G. Millar, M. Unno, H. Hara, T. Calzascia, S. Yamasaki, T. Yokosuka, N.J. Chen, A.R. Elford, et al. 2006. A critical role for the innate immune signaling molecule IRAK-4 in T cell activation. *Science*. 311:1927–1932.

33. Day, N., N. Tangsinmankong, H. Ochs, R. Rucker, C. Picard, J.L. Casanova, S. Haraguchi, and R. Good. 2004. Interleukin receptor-associated kinase (IRAK-4) deficiency associated with bacterial infections and failure to sustain antibody responses. *J. Pediatr.* 144:524–526.
34. Enders, A., U. Pannicke, R. Berner, P. Henneke, K. Radlinger, K. Schwarz, and S. Ehl. 2004. Two siblings with lethal pneumococcal meningitis in a family with a mutation in interleukin-1 receptor-associated kinase 4. *J. Pediatr.* 145:698–700.
35. Medvedev, A.E., K. Thomas, A. Awomoyi, D.B. Kuhns, J.I. Gallin, X. Li, and S.N. Vogel. 2005. Cutting edge: expression of IL-1 receptor-associated kinase-4 (IRAK-4) proteins with mutations identified in a patient with recurrent bacterial infections alters normal IRAK-4 interaction with components of the IL-1 receptor complex. *J. Immunol.* 174:6587–6591.
36. Lavine, E., R. Somech, J.Y. Zhang, A. Puel, X. Bossuyt, C. Picard, J.L. Casanova, and C.M. Roifman. 2007. Cellular and humoral aberrations in a kindred with IL-1 receptor-associated kinase 4 deficiency. *J. Allergy Clin. Immunol.* DOI:10.1016/j.jaci.2007.04.038.
37. Janeway, C.A., Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annu. Rev. Immunol.* 20:197–216.
38. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annu. Rev. Immunol.* 21:335–376.
39. Beutler, B., Z. Jiang, P. Georgel, K. Crozat, B. Croker, S. Rutschmann, X. Du, and K. Hoebe. 2006. Genetic analysis of host resistance: Toll-like receptor signaling and immunity at large. *Annu. Rev. Immunol.* 24: 353–389.
40. Qin, J., Z. Jiang, Y. Qian, J.L. Casanova, and X. Li. 2004. IRAK4 kinase activity is redundant for interleukin-1 (IL-1) receptor-associated kinase phosphorylation and IL-1 responsiveness. *J. Biol. Chem.* 279:26748–26753.
41. Kim, T.W., K. Staschke, K. Bulek, J. Yao, K. Peters, K.H. Oh, Y. Vandenburg, H. Xiao, W. Qian, T. Hamilton, et al. 2007. A critical role for IRAK4 kinase activity in Toll-like receptor–mediated innate immunity. *J. Exp. Med.* 204:1025–1036.
42. Zhang, S.Y., E. Jouanguy, S. Ugolini, A. Smahi, G. Elain, P. Romero, D. Segal, V. Sancho-Shimizu, L. Lorenzo, A. Puel, et al. 2007. TLR3 deficiency in patients with herpes simplex encephalitis. *Science.* 317:1522–1527.
43. Takeuchi, O., K. Hoshino, and S. Akira. 2000. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. *J. Immunol.* 165:5392–5396.
44. Skerrett, S.J., H.D. Liggitt, A.M. Hajjar, and C.B. Wilson. 2004. Cutting edge: myeloid differentiation factor 88 is essential for pulmonary host defense against *Pseudomonas aeruginosa* but not *Staphylococcus aureus*. *J. Immunol.* 172:3377–3381.
45. Albiger, B., A. Sandgren, H. Katsuragi, U. Meyer-Hoffert, K. Beiter, F. Wartha, M. Hornef, S. Normark, and B.H. Normark. 2005. Myeloid differentiation factor 88-dependent signalling controls bacterial growth during colonization and systemic pneumococcal disease in mice. *Cell. Microbiol.* 7:1603–1615.
46. Khan, A.Q., Q. Chen, Z.Q. Wu, J.C. Paton, and C.M. Snapper. 2005. Both innate immunity and type 1 humoral immunity to *Streptococcus pneumoniae* are mediated by MyD88 but differ in their relative levels of dependence on Toll-like receptor 2. *Infect. Immun.* 73:298–307.
47. Delale, T., A. Paquin, C. Asselin-Paturel, M. Dalod, G. Brizard, E.E. Bates, P. Kastner, S. Chan, S. Akira, A. Vicari, et al. 2005. MyD88-dependent and -independent murine cytomegalovirus sensing for IFN-alpha release and initiation of immune responses in vivo. *J. Immunol.* 175:6723–6732.
48. Mansur, D.S., E.G. Kroon, M.L. Nogueira, R.M.E. Arantes, S.C.O. Rodrigues, S. Akira, R.T. Gazzinelli, and M.A. Campos. 2005. Lethal encephalitis in myeloid differentiation factor 88-deficient mice infected with herpes simplex virus 1. *Am. J. Pathol.* 166:1419–1426.
49. Edelson, B.T., and E.R. Unanue. 2002. MyD88-dependent but Toll-like receptor 2-independent innate immunity to *Listeria*: no role for either in macrophage listericidal activity. *J. Immunol.* 169:3869–3875.
50. Seki, E., H. Tsutsui, N.M. Tsuji, N. Hayashi, K. Adachi, H. Nakano, S. Futatsugi-Yumikura, O. Takeuchi, K. Hoshino, S. Akira, et al. 2002. Critical roles of myeloid differentiation factor 88-dependent proinflammatory cytokine release in early phase clearance of *Listeria monocytogenes* in mice. *J. Immunol.* 169:3863–3868.
51. Feng, C.G., C.A. Scanga, C.M. Collazo-Custodio, A.W. Cheever, S. Hieny, P. Caspar, and A. Sher. 2003. Mice lacking myeloid differentiation factor 88 display profound defects in host resistance and immune responses to *Mycobacterium avium* infection not exhibited by Toll-like receptor 2 (TLR2)- and TLR4-deficient animals. *J. Immunol.* 171:4758–4764.
52. Scanga, C.A., J. Aliberti, D. Jankovic, F. Tilloy, S. Bennouna, E.Y. Denkers, R. Medzhitov, and A. Sher. 2002. Cutting edge: MyD88 is required for resistance to *Toxoplasma gondii* infection and regulates parasite-induced IL-12 production by dendritic cells. *J. Immunol.* 168:5997–6001.
53. Yauch, L.E., M.K. Mansour, S. Shoham, J.B. Rottman, and S.M. Levitz. 2004. Involvement of CD14, Toll-like receptors 2 and 4, and MyD88 in the host response to the fungal pathogen *Cryptococcus neoformans* in vivo. *Infect. Immun.* 72:5373–5382.
54. Bellocchio, S., C. Montagnoli, S. Bozza, R. Gaziano, G. Rossi, S.S. Mambula, A. Vecchi, A. Mantovani, S.M. Levitz, and L. Romani. 2004. The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens in vivo. *J. Immunol.* 172:3059–3069.
55. van der Aar, A.M., R.M. Sylva-Steenland, J.D. Bos, M.L. Kapsenberg, E.C. de Jong, and M.B. Teunissen. 2007. Loss of TLR2, TLR4, and TLR5 on Langerhans cells abolishes bacterial recognition. *J. Immunol.* 178:1986–1990.
56. Faure, E., O. Equils, P.A. Sieling, L. Thomas, F.X. Zhang, C.J. Kirschning, N. Polentarutti, M. Muzio, and M. Arditi. 2000. Bacterial lipopolysaccharide activates NF-kappaB through Toll-like receptor 4 (TLR-4) in cultured human dermal endothelial cells. Differential expression of TLR-4 and TLR-2 in endothelial cells. *J. Biol. Chem.* 275:11058–11063.
57. Spachidou, M.P., E. Bourazopoulou, C.I. Maratheftis, E.K. Kapsogeorgou, H.M. Moutsopoulos, A.G. Tzioufas, and M.N. Manoussakis. 2007. Expression of functional Toll-like receptors by salivary gland epithelial cells: increased mRNA expression in cells derived from patients with primary Sjogren's syndrome. *Clin. Exp. Immunol.* 147:497–503.
58. Greene, C.M., and N.G. McElvaney. 2005. Toll-like receptor expression and function in airway epithelial cells. *Arch. Immunol. Ther. Exp. (Warsz.).* 53:418–427.
59. Bozza, S., F. Bistoni, R. Gaziano, L. Pitzurra, T. Zelante, P. Bonifazi, K. Perruccio, S. Bellocchio, M. Neri, A.M. Iorio, et al. 2006. Pentraxin 3 protects from MCMV infection and reactivation through TLR sensing pathways leading to IRF3 activation. *Blood.* 108:3387–3396.
60. Casanova, J.L., and L. Abel. 2004. The human model: a genetic dissection of immunity to infection in natural conditions. *Nat. Rev. Immunol.* 4:55–66.
61. Casanova, J.-L., and L. Abel. 2007. Human genetics of infectious diseases: a unified theory. *EMBO J.* 26:915–922.
62. Casanova, J.L., and L. Abel. 2002. Genetic dissection of immunity to mycobacteria: the human model. *Annu. Rev. Immunol.* 20:581–620.
63. Casrouge, A., S.Y. Zhang, C. Eidenschenk, E. Jouanguy, A. Puel, K. Yang, A. Alcais, C. Picard, N. Mahfoufi, N. Nicolas, et al. 2006. Herpes simplex virus encephalitis in human UNC-93B deficiency. *Science.* 314:308–312.
64. Hultgren, O.H., L. Svensson, and A. Tarkowski. 2002. Critical role of signaling through IL-1 receptor for development of arthritis and sepsis during *Staphylococcus aureus* infection. *J. Immunol.* 168:5207–5212.
65. Zwijnenburg, P.J., T. van der Poll, S. Florquin, J.J. Roord, and A.M. Van Furth. 2003. IL-1 receptor type 1 gene-deficient mice demonstrate an impaired host defense against pneumococcal meningitis. *J. Immunol.* 170:4724–4730.
66. Koedel, U., B. Angele, T. Rupprecht, H. Wagner, A. Roggenkamp, H.W. Pfister, and C.J. Kirschning. 2003. Toll-like receptor 2 participates in mediation of immune response in experimental pneumococcal meningitis. *J. Immunol.* 170:438–444.
67. Malley, R., P. Henneke, S.C. Morse, M.J. Cieslewicz, M. Lipsitch, C.M. Thompson, E. Kurt-Jones, J.C. Paton, M.R. Wessels, and D.T. Golenbock. 2003. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc. Natl. Acad. Sci. USA.* 100:1966–1971.
68. Branger, J., S. Knapp, S. Weijer, J.C. Leemans, J.M. Pater, P. Speelman, S. Florquin, and T. van der Poll. 2004. Role of Toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice. *Infect. Immun.* 72:788–794.

69. Knapp, S., C.W. Wieland, C. van 't Veer, O. Takeuchi, S. Akira, S. Florquin, and T. van der Poll. 2004. Toll-like receptor 2 plays a role in the early inflammatory response to murine pneumococcal pneumonia but does not contribute to antibacterial defense. *J. Immunol.* 172:3132–3138.
70. Dessing, M.C., K.F. van der Sluijs, S. Florquin, S. Akira, and T. van der Poll. 2007. Toll-like receptor 2 does not contribute to host response during postinfluenza pneumococcal pneumonia. *Am. J. Respir. Cell Mol. Biol.* 36:609–614.
71. von Bernuth, H., A. Puel, C.L. Ku, K. Yang, J. Bustamante, H.H. Chang, C. Picard, and J.L. Casanova. 2005. Septicemia without sepsis: inherited disorders of nuclear factor-kappa B-mediated inflammation. *Clin. Infect. Dis.* 41(Suppl. 7):S436–S439.
72. Suresh, M.V., S.K. Singh, D.A. Ferguson Jr., and A. Agrawal. 2007. Human C-reactive protein protects mice from *Streptococcus pneumoniae* infection without binding to pneumococcal C-polysaccharide. *J. Immunol.* 178:1158–1163.
73. Suresh, M.V., S.K. Singh, D.A. Ferguson Jr., and A. Agrawal. 2006. Role of the property of C-reactive protein to activate the classical pathway of complement in protecting mice from pneumococcal infection. *J. Immunol.* 176:4369–4374.
74. Casanova, J.L., and L. Abel. 2005. Inborn errors of immunity to infection: the rule rather than the exception. *J. Exp. Med.* 202:197–201.
75. Kawagoe, T., S. Sato, A. Jung, M. Yamamoto, K. Matsui, H. Kato, S. Uematsu, O. Takeuchi, and S. Akira. 2007. Essential role of IRAK-4 protein and its kinase activity in Toll-like receptor–mediated immune responses but not in TCR signaling. *J. Exp. Med.* 204:1013–1024.
76. Janssen, E., K. Tabeta, M.J. Barnes, S. Rutschmann, S. McBride, K.S. Bahjat, S.P. Schoenberger, A.N. Theofilopoulos, B. Beutler, and K. Hoebe. 2006. Efficient T cell activation via a Toll-interleukin 1 receptor-independent pathway. *Immunity.* 24:787–799.
77. Gavin, A.L., K. Hoebe, B. Duong, T. Ota, C. Martin, B. Beutler, and D. Nemazee. 2006. Adjuvant-enhanced antibody responses in the absence of Toll-like receptor signaling. *Science.* 314:1936–1938.
78. Hirsch, M.S., B. Zisman, and A.C. Allison. 1970. Macrophages and age-dependent resistance to herpes simplex virus in mice. *J. Immunol.* 104:1160–1165.
79. Pham, L.N., M.S. Dionne, M. Shirasu-Hiza, and D.S. Schneider. 2007. A specific primed immune response in *Drosophila* is dependent on phagocytes. *PLoS Pathog.* 3:e26.
80. Filipe-Santos, O., J. Bustamante, M.H. Haverkamp, E. Vinolo, C.L. Ku, A. Puel, D.M. Frucht, K. Christel, H. von Bernuth, E. Jouanguy, et al. 2006. X-linked susceptibility to mycobacteria is caused by mutations in NEMO impairing CD40-dependent IL-12 production. *J. Exp. Med.* 203:1745–1759.

Involvement of Regulatory T Cells in the Experimental Autoimmune Encephalomyelitis-Preventive Effect of Dendritic Cells Expressing Myelin Oligodendrocyte Glycoprotein plus TRAIL[1]

Shinya Hirata, Hidetake Matsuyoshi, Daiki Fukuma, Akari Kurisaki, Yasushi Uemura, Yasuharu Nishimura,[2] and Satoru Senju[2,3]

We previously reported the protection from myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) by the adoptive transfer of genetically modified embryonic stem cell-derived dendritic cells (ES-DC) presenting MOG peptide in the context of MHC class II molecules and simultaneously expressing TRAIL (ES-DC-TRAIL/MOG). In the present study, we found the severity of EAE induced by another myelin autoantigen, myelin basic protein, was also decreased after treatment with ES-DC-TRAIL/MOG. This preventive effect diminished, if the function of $CD4^+CD25^+$ regulatory T cells (Treg) was abrogated by the injection of anti-CD25 mAb into mice before treatment with ES-DC-TRAIL/MOG. The adoptive transfer of $CD4^+CD25^+$ T cells from ES-DC-TRAIL/MOG-treated mice protected the recipient mice from MOG- or myelin basic protein-induced EAE. The number of $Foxp3^+$ cells increased in the spinal cords of mice treated with ES-DC-TRAIL/MOG. In vitro experiments showed that TRAIL expressed in genetically modified ES-DC and also in LPS-stimulated splenic macrophages had a capacity to augment the proliferation of $CD4^+CD25^+$ T cells. These results suggest that the prevention of EAE by treatment with ES-DC-TRAIL/MOG is mediated, at least in part, by MOG-reactive $CD4^+CD25^+$ Treg propagated by ES-DC-TRAIL/MOG. For the treatment of organ-specific autoimmune diseases, induction of Treg reactive to the organ-specific autoantigens by the transfer of DC-presenting Ags and simultaneously overexpressing TRAIL therefore appears to be a promising strategy. *The Journal of Immunology,* **2007, 178: 918–925.**

For the treatment of subjects with autoimmune and allergic diseases, it is desirable to down-modulate the immune response in an Ag-specific manner while not causing systemic immune suppression. To achieve this goal, genetically modified dendritic cells (DC)[4] presenting target Ags and simultaneously expressing immunoinhibitory molecules would be an attractive strategy (1).

TRAIL, a member of the TNF superfamily, is expressed in a variety of cell types, including lymphocytes, NK cells, NKT cells, and virus-infected APCs (2–5). The abrogation of functional TRAIL by gene targeting or the in vivo administration of soluble death receptor 5, one of receptors for TRAIL, results in the acceleration of autoimmune diseases in mouse models, for example collagen-induced arthritis, autoimmune diabetes, and experimental autoimmune encephalomyelitis (EAE) (6–9). It is thus evident that TRAIL plays a critical role in the regulation of the immune response or the maintenance of immunological self-tolerance to prevent autoimmunity. However, the precise mechanism for this has not yet been clarified regarding how TRAIL exerts such an effect.

We recently reported the protection from myelin oligodendrocyte glycoprotein (MOG)-induced EAE with genetically modified DC expressing MOG peptide along with TRAIL or programmed death-1 ligand (PD-L1) (1). For the genetic modification of DC, we used a method to generate DC from mouse embryonic stem cells in vitro (ES-DC) (10–13). For the efficient presentation of MOG peptide in the context of MHC class II molecules, we used an expression vector in which cDNA encoding for human MHC class II-associated invariant chain was mutated to contain antigenic peptide in the class II-associated invariant chain peptide region (14, 15). An epitope inserted into this vector is efficiently presented in the context of coexpressed MHC class II molecules. Based on these technologies, we generated transfectant ES-DC presenting MOG peptide and simultaneously expressing TRAIL or PD-L1, ES-DC-TRAIL/MOG, and ES-DC-PDL1/MOG, respectively.

The treatment of mice with either of the double-transfectant ES-DC significantly reduced the severity of MOG-induced EAE. In contrast, treatment with ES-DC expressing MOG alone, irrelevant Ag (OVA) plus TRAIL, or OVA plus PD-L1, or coinjection with ES-DC expressing MOG plus ES-DC expressing TRAIL or PD-L1, had no effect on the disease course. The immune response to irrelevant exogenous Ag (keyhole limpet hemocyanin) was not

Department of Immunogenetics, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan

Received for publication March 9, 2006. Accepted for publication October 31, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by Grants-in-Aid 12213111, 14370115, 14570421, 14657082, and the Program of Founding Research Centers for Emerging and Re-emerging Infectious Disease from the Ministry of Education, Science, Technology, Sports, and Culture, Japan, and a Research Grant for Intractable Diseases from the Ministry of Health, Labour and Welfare, Japan, and grants from the Uehara Memorial Foundation, and by funding from the Meiji Institute of Health Science.

[2] Y.N. and S.S. contributed equally to this study.

[3] Address correspondence and reprint requests to Dr. Satoru Senju, Department of Immunogenetics, Graduate School of Medical Sciences, Kumamoto University, Honjo 1-1-1, Kumamoto 860-8556, Japan. E-mail address: senjusat@gpo.kumamoto-u.ac.jp

[4] Abbreviations used in this paper: DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; ES, embryonic stem cell; PD-L1, programmed death-1 ligand; MBP, myelin basic protein; Treg, regulatory T cell; Tr1, T regulatory type 1.

 0022-1767/07/$2.00

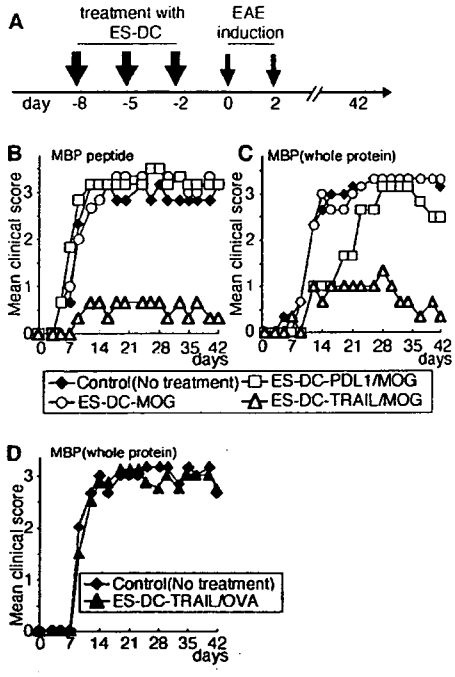


FIGURE 1. Prevention of MBP-induced EAE by treating the mice with ES-DC-TRAIL/MOG. *A*, The schedule for the pretreatment and induction of EAE is shown. CBF_1 mice (three to four mice per group) were i.p. injected with ES-DC (1×10^6 cells/mouse/injection) on days −8, −5, and −2. EAE was induced by the immunization of MBP peptide or whole protein on day 0, and the injection of *B. pertussis* toxin on days 0 and 2. *B–D*, The disease severity of mice immunized with MBP peptide (*B*) or whole protein (*C* and *D*) is shown. The data of all experiments are summarized in Table I.

impaired by treatment with any of the genetically modified ES-DC. These results suggest the possibility of treating autoimmune diseases without affecting immunity to exogenous Ags using genetically engineered DC presenting target autoantigen and simultaneously expressing TRAIL or PD-L1. In that study, we observed an increase in apoptosis of $CD4^+$ T cells in the spleens of mice treated with ES-DC-TRAIL/MOG, suggesting that protection from EAE by ES-DC-TRAIL/MOG is mediated by induction of apoptosis of MOG-reactive pathogenic $CD4^+$ T cells. In the present study, we found that the severity of not only MOG- but also myelin basic protein (MBP)-induced EAE was reduced by treatment with ES-DC-TRAIL/MOG. Regarding the mechanism underlying this disease-preventive effect, we obtained several lines of evidence supporting that MOG-reactive $CD4^+CD25^+$ regulatory T cells (Treg) were activated or propagated by the transfer of ES-DC-TRAIL/MOG and that the prevention of EAE by treatment with ES-DC-TRAIL/MOG was mediated, at least in part, by Treg.

Materials and Methods

Mice and cells

CBA and C57BL/6 mice obtained from Clea Animal or Charles River Laboratories were kept under specific pathogen-free conditions. Male CBA and female C57BL/6 mice were mated to generate F_1 (CBF_1) mice and all in vivo experiments were done using CBF_1 mice, syngeneic to TT2 ES cells. The mouse experiments were approved by the Animal Research Committee of Kumamoto University.

The mouse ES cell line, TT2, derived from CBF_1 blastocysts, and OP9 were maintained as previously described (10). The induction of differentiation of ES cells into ES-DC and generation of transfectant ES-DC-TRAIL, ES-DC-PDL1, ES-DC-MOG, ES-DC-TRAIL/MOG, ES-DC-PDL1/MOG, and ES-DC-TRAIL/OVA was done as described previously (1).

Protein, cytokines, and Abs

The mouse MOG p35–55 (MEVGWYRSPFSRVVHLYRNGK) and mouse MBP p35-47 (TGILDSIGRFFSG) were synthesized using the F-moc method on an automatic peptide synthesizer (PSSM8; Shimadzu) and purified using HPLC. Recombinant mouse GM-CSF (PeproTech) was purchased. Rat anti-mouse CD25 mAb was produced by culturing the PC61.5.3 cell line in the CELLINE system (BD Biosciences) and was purified by the MAbTrap kit (Amersham Biosciences). Abs and reagents used for staining were PE-conjugated anti-mouse CD25 (clone 3C7, rat IgG2b; BD Pharmingen) and FITC-conjugated anti-mouse CD4 (clone GK1.5, rat IgG2b; BD Pharmingen).

Induction of EAE and treatment with ES-DC

For the induction of EAE, 6- to 8-wk-old female CBF_1 mice were immunized by performing a s.c. injection at the base of tail with a 0.2-ml IFA/PBS solution containing 600 μg of MOG p35–55 peptide, MBP p35–47 peptide, or whole bovine MBP (Sigma-Aldrich), and 400 μg of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories) on day 0. In addition, 500 ng of purified *Bordetella pertussis* toxin (Calbiochem) was injected i.p. on days 0 and 2 (1). For the prevention of EAE, mice were injected i.p. with ES-DC (1×10^6 cells/mouse/injection) on days −8, −5, and −2 (preimmunization treatment), or on days 14, 17, and 21 (postonset treatment). In some experiments, $CD25^+$ T cells were depleted by i.p. injections of anti-mouse CD25 mAb (clone PC61.5.3) as described (16). In brief, the mAb (400 μg/mouse) was administrated on days −28, −24, −21, and −14. Depletion was verified by staining PBMC and then analyzing them on a flow cytometer (FACScan; BD Biosciences). The mice were observed over a period of 42 or 56 days (postonset treatment) for clinical signs and scores were assigned based on the following scale: 0, normal; 1, weakness of the tail and/or paralysis of the distal half of the tail; 2, loss of tail tonicity and abnormal gait; 3, weakly partial hind-limb paralysis; 3.5, strongly partial

Table I. *Suppression of MBP-induced EAE induction in CBF_1 mice treated with ES-DC expressing MOG plus TRAIL*[a]

Treatment (ES-DC) of Mice	EAE Induced with	Disease Incidence	Day of Onset	Mean Peak Clinical Score
No treatment (control)	MBP pep	12/12	6.4 ± 1.3	3.1 ± 0.1
TRAIL/MOG	MBP pep	2/6	8.0 ± 1.0	0.7 ± 0.9
PD-L1/MOG	MBP pep	6/6	8.0 ± 2.7	3.4 ± 0.1
MOG	MBP pep	3/3	6.3 ± 1.8	3.5 ± 0.0
No treatment (control)	MBP whole	15/15	9.4 ± 1.5	3.1 ± 0.2
TRAIL/MOG	MBP whole	5/9	15.2 ± 4.6	1.0 ± 0.9
PD-L1/MOG	MBP whole	6/6	13.5 ± 3.3	3.1 ± 0.1
MOG	MBP whole	6/6	11.7 ± 0.6	3.2 ± 0.2
TRAIL/OVA	MBP whole	4/4	9.8 ± 1.1	3.3 ± 0.3
CD25 depl.[b] plus no treatment	MBP whole	6/6	10.1 ± 1.2	3.8 ± 0.5
CD25 depl.[b] plus TRAIL/MOG	MBP whole	8/8	10.0 ± 1.5	4.3 ± 1.0

[a] The data are combined from a total of nine separate experiments including those shown in Figs. 1 and 4.

[b] In these mice, $CD25^+$ cells were depleted by the treatment with anti-CD25 mAb and subsequently mice were transferred with ES-DC or left untreated. The values of onset day and mean peak clinical score are rounded off to the first decimal place.

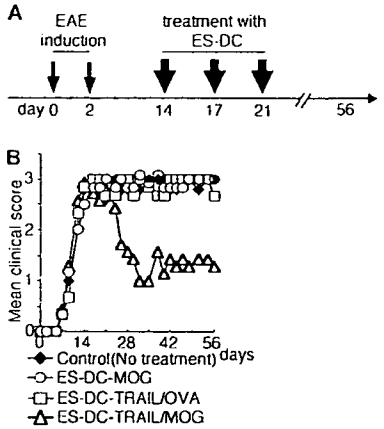


FIGURE 2. Inhibition of MBP-induced EAE by treating the mice with ES-DC-TRAIL/MOG. *A*, The schedule for induction of EAE and treatment is shown. CBF_1 mice (five to seven mice per group) were immunized on days 0 and 2 according to the EAE-induction schedule described above, and subsequently i.p. injected with ES-DC (1×10^6 cells/mouse/injection) on days 14, 17, and 21. *B*, The disease severity of mice immunized with MBP whole protein is shown.

hind-limb paralysis; 4, complete hind-limb paralysis; 5, fore-limb paralysis or moribundity; 6, death.

Adoptive transfer of T cells

For the adoptive transfer experiments, donor CBF_1 mice were i.p. injected with ES-DC (1×10^6 cells/injection/mouse) on days −10, −7, and −4. $CD4^+$ T cells and $CD4^+CD25^+$ T cells were isolated from the spleen cells of donor mice using the MACS cell sorting system (Miltenyi Biotec). For the isolation of $CD4^+$ T cells, non-$CD4^+$ T cells magnetically labeled with a biotin-conjugated Ab mixture (anti-CD8α, anti-CD11b, anti-CD45R, anti-DX5, and anti-Ter-119) and anti-biotin microbeads were depleted on an autoMACS cell separator. Subsequently, $CD25^+$ T cells, labeled with anti-CD25 mAb conjugated with PE and anti-PE microbeads, were isolated from the $CD4^+$ T cell fraction using positive sorting columns. Cell purity was checked by FACS analysis: $CD4^+$ T cells were >95% after the first step and $CD4^+CD25^+$ cells were >95% after the second step. The $CD4^+$ T cells, $CD4^+CD25^+$ T cells, or $CD4^+CD25^-$ T cells were i.v. injected into recipient mice (2.5×10^6, 3×10^5, or 2.2×10^6 cells/mouse, respectively) on day −2. The recipient mice were subjected to EAE induction (on days 0 and 2) as described above.

Proliferation assay of Treg

Mouse $CD4^+CD25^+$ Treg were purified with a Treg separation kit (MACS) from the spleen cells of naive CBF_1 mice as described above. Assay for the proliferation of Treg was done, as described previously (17). In brief, 1×10^4 ES-DC or syngeneic splenic macrophages were x-ray irradiated (25 Gy), and cocultured with 1×10^4 Treg in the presence of anti-CD3 mAb (clone 145-2C11, 1 μg/ml) and human IL-2 (10–30 U/ml) in wells of 96-well round-bottom culture plates. In some assay, anti-TRAIL mAb (clone N2B2 (5 μg/ml); eBioscience) was added. Splenic macrophages were prepared by collecting plastic dish-adherent cells. The cells were cultured for 3 days, and [^{3}H]thymidine (6.7 Ci/mM) was added to the culture (1 μCi/well) in the last 12 h. At the end of culture, cells were harvested onto glass fiber filters (Wallac) and the incorporation of [^{3}H]thymidine was measured by scintillation counting. The expression of TRAIL in LPS-stimulated spleen cells and macrophages was confirmed by RT-PCR, as described previously (1). The relative quantity of cDNA in each sample was first normalized by PCR for G3PDH. The primer sequences were as follows: TRAIL, 5′-AACCCTCTAGACCGCCGCCACCATGCCTTCCTCAGGGG CCCTGAA-3′ and 5′-GAAATGGTGTCCTGAAAGGTTC; G3PDH, 5′-GG AAAGCTGTGGCGTGATG-3′ and 5′-CTGTTGCTGTAGCCGTATTC-3′.

Immunohistochemical analysis

Freshly excised spinal cords were immediately frozen and embedded in Tissue-Tek OCT compound (Sakura Fine Technical). Immunohistochemical staining of Foxp3 and CD4 was done, as previously described (1, 12), but with some modification. In brief, serial 7-μm sections were made using cryostat and underwent immunochemical staining with anti-Foxp3 mAb

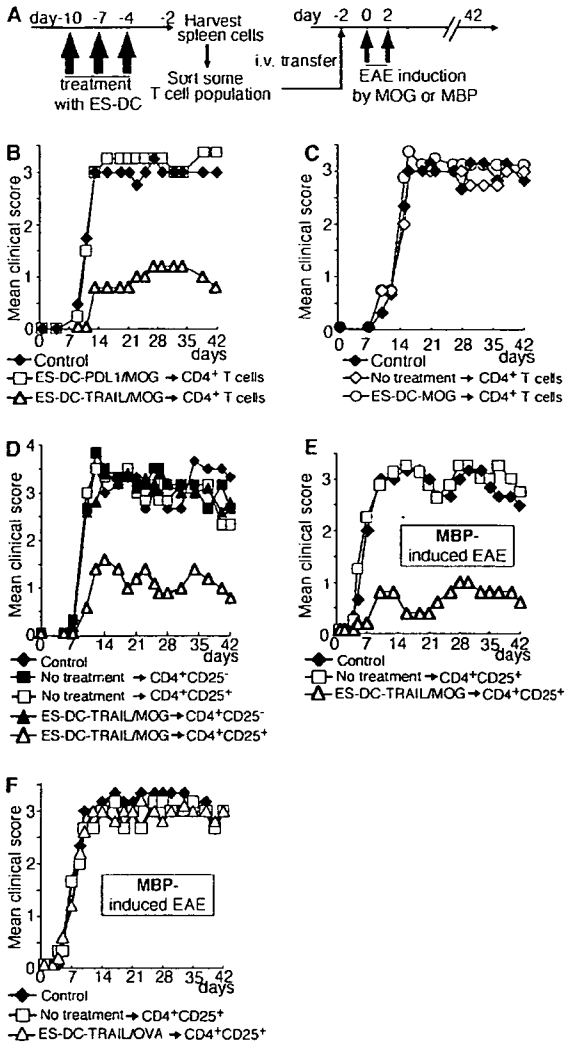


FIGURE 3. Protection from MOG or MBP-induced EAE by the adoptive transfer of $CD4^+CD25^+$ T cells from the mice treated with ES-DC expressing MOG plus TRAIL. *A*, The schedule for the treatment of donor mice with ES-DC, the adoptive transfer of some population of T cells, and the induction of EAE in the recipient mice are shown. The donor CBF_1 mice were i.p. injected with ES-DC (1×10^6 cells/mouse/injection) on days −10, −7, and −4. The T cells were isolated from the donor mice and then transferred to naive mice on day −2. The recipient mice were immunized with MOG peptide (*B–D*) or MBP whole protein (*E* and *F*) on days 0 and 2 according to the EAE-induction protocol as shown in Fig. 1. *B* and *C*, The disease severity of the mice transferred with $CD4^+$ T cells (2.5×10^6 cells/mouse) from ES-DC-TRAIL/MOG, ES-DC-PD-L1/MOG, ES-DC-MOG, ES-DC-TRAIL/OVA-treated mice, or naive mice is shown. *D–F*, The disease severity of the mice transferred with $CD4^+CD25^+$ T cells or $CD4^+CD25^-$ T cells (3×10^5 or 2.2×10^6 cells/mouse, respectively) from ES-DC-TRAIL/MOG-treated mice or naive mice is shown. Control mice were injected with RPMI 1640 medium alone without T cells. The data of all experiments are summarized in Table II.

(clone FJK-16s, rat IgG2a; eBioscience) or CD4 (clone L3T4; BD Pharmingen), and N-Histofine Simple Stain Mouse MAX PO (Nichirei).

Statistical analysis

The two-tailed Student's *t* test was used to determine any statistical significance of differences. A value of $p < 0.05$ was considered to indicate statistical significance (15). The values indicated in tables are rounded off to the first decimal place.

Table II. *Suppression of EAE induction by adoptive transfer of T cells from CBF$_1$ mice treated with ES-DC expressing MOG plus TRAIL*[a]

Treatment (ES-DC) of Donor Mice	T Cells Transferred	EAE Induction	Disease Incidence	Day of Onset	Mean Peak Clinical Score
	No transfer	MOG pep	14/14	10.3 ± 1.3	3.6 ± 0.4
TRAIL/MOG	$CD4^+$	MOG pep	3/7	12.3 ± 0.9	1.0 ± 1.1
PD-L1/MOG	$CD4^+$	MOG pep	4/4	10.5 ± 0.8	3.4 ± 0.2
MOG	$CD4^+$	MOG pep	4/4	11.3 ± 1.9	3.4 ± 0.4
No treatment	$CD4^+$	MOG pep	4/4	11.3 ± 1.9	3.0 ± 0.0
TRAIL/MOG	$CD4^+CD25^+$	MOG pep	5/7	14.4 ± 4.2	1.7 ± 1.0
TRAIL/MOG	$CD4^+CD25^-$	MOG pep	7/7	9.0 ± 1.1	3.4 ± 0.2
No treatment	$CD4^+CD25^+$	MOG pep	3/3	9.0 ± 1.3	3.7 ± 0.2
No treatment	$CD4^+CD25^-$	MOG pep	3/3	9.0 ± 1.3	3.8 ± 0.2
	No transfer	MBP pep	18/18	6.4 ± 1.3	3.1 ± 0.1
TRAIL/MOG	$CD4^+CD25^+$	MBP pep	3/8	13.0 ± 8.7	0.8 ± 0.9
TRAIL/OVA	$CD4^+CD25^+$	MBP pep	8/8	6.1 ± 1.4	3.1 ± 0.2
No treatment	$CD4^+CD25^+$	MBP pep	7/7	5.7 ± 1.1	3.1 ± 0.1

[a] The data are combined from a total of 10 separate experiments, including those shown in Fig. 3. The values of onset day and mean peak clinical score are rounded off to the first decimal place.

Results

Prevention of MBP-induced EAE by the transfer of ES-DC genetically engineered to express MOG peptide along with TRAIL

We recently demonstrated the prevention of MOG-induced EAE by treatment with ES-DC expressing MOG peptide plus TRAIL (ES-DC-TRAIL/MOG) or MOG peptide plus PD-L1 (ES-DC-PDL1/MOG). Regarding the mechanism for preventing EAE by the genetically modified ES-DC, we considered not only the possibility of the direct down-modulation of MOG-reactive effector T cells such as the induction of anergy or apoptosis, but also the possibility of promoting MOG-reactive T cells with regulatory or immune-suppressive functions. We hypothesized that, if the latter had been the case, then pretreatment with ES-DC-TRAIL/MOG or ES-DC-PDL1/MOG may thus have had some preventive effect on not only MOG- but also MBP-induced EAE. To test this possibility, we pretreated mice with ES-DC-TRAIL/MOG or ES-DC-PDL1/MOG and subjected them to EAE induction by immunization with MBP (whole protein) or MBP p35-47, according to the schedule depicted in Fig. 1*A*. As a result, we found the severity of both MBP whole protein- and peptide-induced EAE to be significantly reduced by pretreatment with ES-DC-TRAIL/MOG. In contrast, pretreatment with ES-DC-PDL1/MOG, ES-DC-TRAIL/OVA (as irrelevant Ag), and ES-DC-MOG had no effect on MBP-induced EAE (Fig. 1, *B–D*, and Table I).

Next, we tested whether treatment with ES-DC after the onset of MBP-induced EAE would achieve some suppressive effect on the course of disease. The mice immunized according to the protocol for MBP-induced EAE were injected with ES-DC on days 14, 17, and 21 (1×10^6 cells/mouse/injection) as shown in Fig. 2*A*. Even in this postonset treatment, injection of ES-DC-TRAIL/MOG reduced severity of the disease, while ES-DC-TRAIL/OVA and ES-DC-MOG did not do so (Fig. 2*B*).

Prevention of MOG- and MBP-induced EAE by the adoptive transfer of $CD4^+CD25^+$ T cells from the mice treated with ES-DC-TRAIL/MOG

We considered the possibility that MOG-reactive T cells possessing some immunoregulatory effect were activated or propagated by the transferred ES-DC-TRAIL/MOG and that the T cells exerted a protective effect against MBP-induced EAE. To address this possibility, we performed adoptive transfer experiments. We isolated $CD4^+$ T cells from the spleens of the donor mice treated with ES-DC and transferred them into naive recipient mice. Subsequently, the recipient mice were subjected to an immunization procedure for MOG-induced EAE (Fig. 3*A*). As shown in Fig. 3, *B* and *C*, and Table II, the transfer of 2.5×10^6 $CD4^+$ T cells isolated from the mice treated with ES-DC-TRAIL/MOG significantly reduced the severity of EAE of the recipient mice. In contrast, $CD4^+$ T cells isolated from the mice treated with ES-DC-PDL1/MOG or ES-DC-MOG or those from untreated mice showed no effect. These results support the notion that $CD4^+$ T cells with some regulatory activity were induced, activated, or propagated by the treatment with ES-DC-TRAIL/MOG.

Several types of $CD4^+$ T cells with a potential for reducing the severity of Th1 cell-mediated autoimmune diseases are known, such as T regulatory type 1 (Tr1) cells, Th2 cells, or $CD4^+CD25^+$ Treg. To identify the type of T cells involved in the disease-preventive effect, we separated $CD4^+$ T cells isolated from ES-DC-TRAIL/MOG-treated mice into $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells before transfer. In CBF$_1$ mice, 10–15% of splenic $CD4^+$ T cells were $CD25^+$ (data not shown), thus indicating that 2.5×10^6 $CD4^+$ T cells include $\sim 3 \times 10^5$ $CD25^+$ T cells and 2.2×10^6 $CD25^-$ T cells. Therefore, we transferred 3×10^5 $CD25^+$ T cells and 2.2×10^6 $CD25^-$ T cells into separate mice. As shown in Fig. 3*D* and Table II, the transfer of 3×10^5 $CD4^+CD25^+$ T cells isolated from mice treated with ES-DC-TRAIL/MOG significantly reduced the severity of MOG-induced EAE in the recipient mice. In contrast, the transfer of $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells from naive mice or $CD4^+CD25^-$ T cells from ES-DC-TRAIL/MOG-treated mice had no effect. These results indicate that $CD25^+$ T cells among the $CD4^+$ T cells were responsible for the above-described protective effect of ES-DC-TRAIL/MOG against EAE.

We next tested whether the transfer of $CD4^+CD25^+$ T cells isolated from donor mice treated with ES-DC-TRAIL/MOG would have any effect on MBP-induced EAE. We transferred $CD4^+CD25^+$ T cells from the donor mice treated with ES-DC as described above. Subsequently, the recipient mice were subjected to an immunization procedure for MBP-induced EAE. As shown in Fig. 3, *E* and *F*, and Table II, the transfer of 3×10^5 $CD4^+CD25^+$ T cells isolated from mice treated with ES-DC-TRAIL/MOG significantly reduced the severity of MBP-induced EAE in the recipient mice, but the transfer of $CD4^+CD25^+$ T cells isolated from ES-DC-TRAIL/OVA-treated mice or naive mice did not do so. These results indicate that ES-DC-TRAIL/MOG-

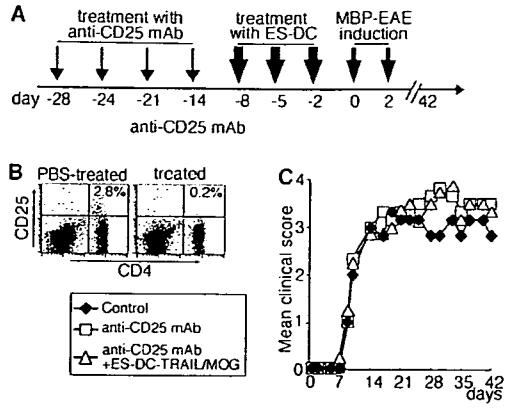


FIGURE 4. The depletion of $CD4^+CD25^+$ T cells diminished the preventive effect of ES-DC-TRAIL/MOG on MBP-induced EAE. *A*, The schedule for the depletion with anti-CD25 mAb, pretreatments with ES-DC, and induction of EAE are shown. CBF_1 mice (three to four mice per group) were i.p. injected with anti-mouse CD25 mAb (400 μg/mouse/injection) on days −28, −24, −21, −14 and then were treated with ES-DC (1×10^6 cells/mouse/injection) on days −8, −5, and −2. EAE was induced by immunization with MBP whole protein on days 0 and 2 as in Fig. 1. *B*, $CD4^+CD25^+$ cells of peripheral blood of anti-CD25 mAb-treated (*right*) and PBS-treated control (*left*) mice were analyzed by flow cytometry on day −2. The percentage of $CD4^+CD25^+$ cells among $CD4^+$ cells is shown. *C*, The severity of MBP-induced EAE is shown. Control mice were injected with PBS alone without Ab and RPMI 1640 medium alone without ES-DC. The data of all experiments are summarized in Table I.

induced $CD4^+CD25^+$ T cells had a protective effect against not only MOG- but also MBP-induced EAE.

Protection from MBP-induced EAE by ES-DC-TRAIL/MOG depends on $CD25^+$ cells

To further verify the involvement of $CD4^+CD25^+$ T cells in the disease-preventive effect of ES-DC-TRAIL/MOG, we performed depletion experiments of $CD4^+CD25^+$ T cells. We injected the mice with anti-mouse CD25 mAb four times on days −28, −24, −21, and −14 (Fig. 4*A*). Depletion of $CD4^+CD25^+$ T cells was confirmed by flow cytometry analysis of PBLs. $CD25^+$ cells were $0.3 \pm 0.1\%$ of $CD4^+$ T cells in anti-CD25 mAb-treated mice ($n = 4$), whereas they were $2.6 \pm 0.3\%$ in control (PBS-injected) mice ($n = 3$). Representative results of flow cytometry analysis are shown in Fig. 4*B*. Thereafter, we treated the mice with ES-DC-TRAIL/MOG and then immunized them with MBP according to the EAE induction protocol. As shown in Fig. 4*C* and Table I, the effect of ES-DC-TRAIL/MOG to prevent MBP-induced EAE completely disappeared in the mice in which the $CD4^+CD25^+$ T cells were depleted. These results further support the possibility that the prevention of MBP-induced EAE by ES-DC-TRAIL/MOG was mediated by $CD4^+CD25^+$ T cells. In addition, treatment with anti-CD25 mAb slightly worsened the disease course even when mice were not treated with ES-DC, suggesting that $CD4^+CD25^+$ T cells ameliorate the disease to some extent in the natural course after EAE induction.

Increased number of $Foxp3^+$ cells infiltrating into spinal cords of mice treated with ES-DC-TRAIL/MOG

At present, Foxp3 is the most reliable molecular marker for Treg (18). We performed immunohistochemical analysis to detect $Foxp3^+$ cells in the spinal cord of mice treated with ES-DC. We treated the mice with ES-DC and then immunized them with MOG according to the EAE induction protocol. On day 11, the spinal cords harvested from mice were stained with anti-Foxp3 mAb (Fig. 5, *A–C*) and anti-CD4 mAb, and $Foxp3^+$ cells and $CD4^+$ cells were counted. As shown in Fig. 5*D*, the infiltration of $Foxp3^+$ cells into the spinal cord was enhanced in mice treated with ES-DC-TRAIL/MOG, compared with mice with no treatment or mice treated with ES-DC-PDL1/MOG. In contrast, the infiltration of $CD4^+$ cells into the spinal cords was reduced in mice treated with ES-DC-TRAIL/MOG or ES-DC-PDL1/MOG, compared with the observation in mice with no treatment. These results further support the notion of involvement of Treg in the disease-protection effect.

Enhanced capacity of ES-DC expressing TRAIL to induce the in vitro proliferation of naive $CD4^+CD25^+$ Tregs

Recent studies have demonstrated that bone marrow-derived DC and splenic DC have a potent capacity to promote the proliferation of $CD4^+CD25^+$ Treg both in vitro and in vivo (17, 19). We investigated whether ES-DC had the capacity to promote the proliferation of $CD4^+CD25^+$ Treg and also whether the expression of TRAIL had any effect on this capacity of ES-DC. Treg isolated from spleen of naive CBF_1 mice were cocultured with ES-DC in the presence of anti-CD3 mAb (1 μg/ml) and a low dose of human IL-2 (10 U/ml). As shown in Fig. 6*A*, all three types of ES-DC, nontransfectant ES-DC, ES-DC-TRAIL, or ES-DC-PDL1, induced a proliferation of Treg more potently than splenic macrophages. The increased proliferation of Treg was observed upon coculture with ES-DC-TRAIL, in comparison to that with other

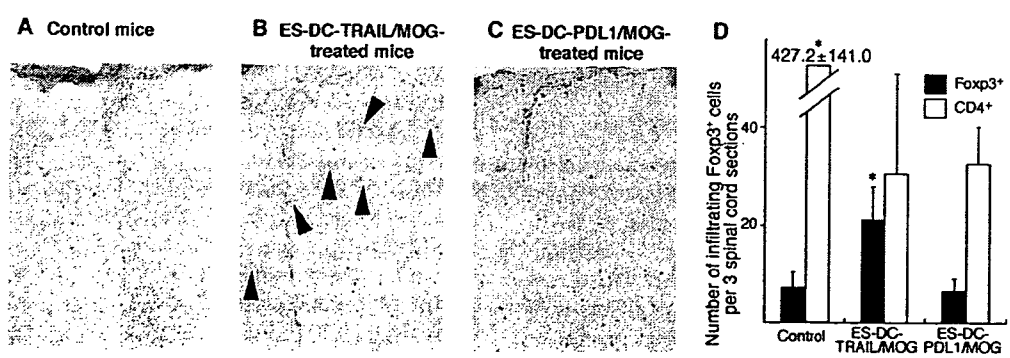


FIGURE 5. Increased number of $Foxp3^+$ cells in spinal cords of mice treated with ES-DC expressing MOG plus TRAIL. CBF_1 mice (five mice per group) were pretreated with ES-DC-TRAIL/MOG or PDL1/MOG (1×10^6 cells/mouse/injection) as in Fig. 1 or left untreated. Subsequently, EAE was induced by immunization with MOG peptide on days 0 and 2. The cervical, thoracic, and lumbar spinal cord was isolated on day 11 and subjected to immunohistochemical analysis. The $Foxp3^+$ cells (*A–C*, arrowhead) and $CD4^+$ cells were stained and microscopically counted in three sections of spinal cord (*D*). Results are expressed as mean of samples obtained from five mice ± SD. *, The increase in number of infiltrated cells is statistically significant ($p < 0.01$) as compared with other groups.

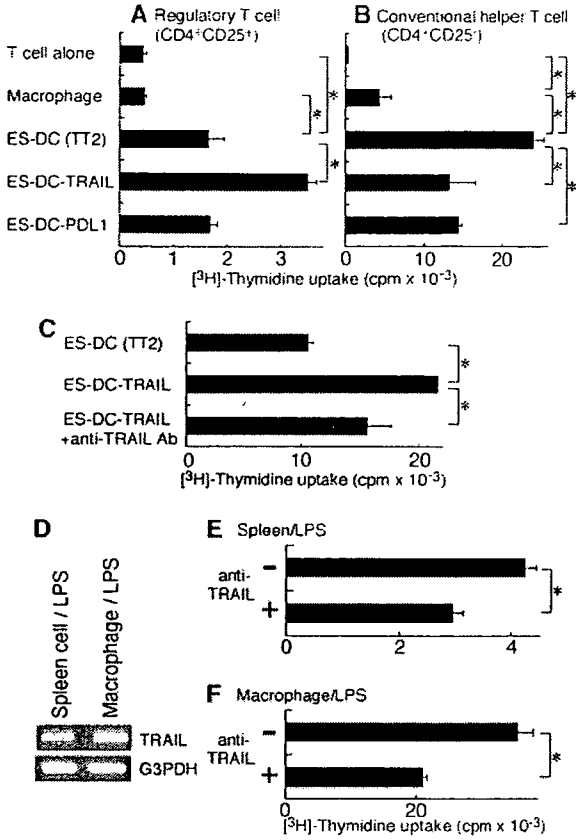


FIGURE 6. Enhanced capacity of APC expressing TRAIL to induce the proliferation of naive $CD4^+CD25^+$ Tregs in vitro. *A*, $CD4^+CD25^+$ T cells (1×10^4) or (*B*) $CD4^+CD25^-$ T cells (1×10^4) isolated from the spleen of the naive CBF_1 mice were cultured alone or cocultured with irradiated stimulators (1×10^4), syngeneic splenic macrophages, nontransfectant ES-DC (TT2), ES-DC-TRAIL, or ES-DC-PDL1, for 3 days in the presence of anti-CD3 mAb (0.1 μg/ml) and human IL-2 (10 U/ml). *C*, $CD4^+CD25^+$ T cells (1×10^4) isolated from the spleen of the naive CBF_1 mice were cultured alone or cocultured with irradiated stimulators (1×10^4), nontransfectant ES-DC (TT2), or ES-DC-TRAIL, for 3 days in the presence of anti-CD3 mAb (2.5 μg/ml) and human IL-2 (10 U/ml) with or without anti-mouse TRAIL mAb (2.5 μg/ml). *D*, The expression of TRAIL in spleen cells or macrophages stimulated with LPS was confirmed by RT-PCR. The naive Treg isolated from a spleen were cocultured for 3 days with 1×10^5 of irradiated LPS-stimulated spleen cells (*E*) or 1×10^4 of irradiated LPS-stimulated macrophages (*F*) in the presence of anti-CD3 mAb (1 μg/ml) and IL-2 (30–100 U/ml) with or without anti-mouse TRAIL mAb (5 μg/ml) for 3 days. The proliferation of responder T cells was quantified by measuring the $[^3H]$thymidine incorporation in the last 12 h of the culture. *, Statistical significance ($p < 0.01$). The results were expressed as the mean of a triplicate assay ± SD. The data are each representative of more than three independent experiments with similar results.

type of ES-DC. In contrast, the magnitude of proliferation of $CD4^+CD25^-$ conventional Th cells was decreased by the expression of TRAIL by ES-DC (Fig. 6*B*). In addition, anti-TRAIL blocking mAb decreased the proliferation of Treg cocultured with ES-DC-TRAIL (Fig. 6*C*). These results suggest that TRAIL expressed on ES-DC has an inhibitory effect on the proliferation of conventional $CD4^+$ T cells and a stimulating effect on that of $CD4^+CD25^+$ Treg.

Anti-TRAIL blocking mAb decreased the proliferation of $CD4^+CD25^+$ Treg responding to LPS-treated natural APCs

The data presented so far suggest that TRAIL expressed on ES-DC has an effect to augment proliferation of $CD4^+CD25^+$ Treg. Lastly, we investigate the effect of TRAIL expressed on natural APCs on proliferation of Treg. Recent studies reported that LPS-stimulation enhanced the expression of TRAIL on spleen cells and bone marrow-derived DC (20–22). We thus examined the proliferation of Treg cocultured with LPS-stimulated whole spleen cells or splenic macrophages in the presence or absence of anti-TRAIL-blocking mAb. Treg isolated from spleen of naive CBF_1 mice were cocultured with LPS-treated APC in the presence of anti-CD3 mAb and a low dose of human IL-2 with or without anti-TRAIL mAb (5 μg/ml). As shown in Fig. 6, *D–F*, anti-TRAIL mAb partially decreased the proliferation of $CD4^+CD25^+$ Treg. These results indicate that TRAIL naturally expressed on APC as well as that expressed on genetically modified ES-DC has a stimulating effect on proliferation of $CD4^+CD25^+$ Treg.

Discussion

In the present study, we found that the severity of not only MOG- but also MBP-induced EAE was reduced by treatment with ES-DC expressing MOG peptide along with TRAIL (Figs. 1 and 2). We obtained several lines of evidences suggesting a possibility that the observed disease-preventive effect is mediated by propagation of MOG-reactive Treg by ES-DC-TRAIL/MOG. Another possible explanation for this phenomenon may be as follows: MOG-reactive T cells might be activated by so-called epitope spreading even in MBP-induced EAE and they might play a major role in the pathogenesis; pretreatment with ES-DC-TRAIL/MOG may abrogate MOG-reactive T cells, thus resulting in a reduction of the disease severity. However, we consider this possibility to be less likely because ES-DC-PDL1/MOG, which showed a MOG-induced EAE-preventive effect similar to that induced by ES-DC-TRAIL/MOG, had no effect on MBP-induced EAE (Fig. 1, *B* and *C*, and Table I). In addition, in previous studies of EAE, the autoreactivity directed to multiple myelin Ags caused by epitope spreading have been observed in chronic or relapsing phases over 4 wk after the immunization. In contrast, the inhibitory effect on MBP-induced EAE, which we observed in the present study, occurred within 2 wk after the immunization. We therefore considered the possibility that MOG-specific T cells with some regulatory activity may have been induced or stimulated by ES-DC-TRAIL/MOG. The results of adoptive transfer experiments demonstrating that $CD4^+$ T cells isolated from ES-DC-TRAIL/MOG-treated donor mice acted to protect the recipient mice from EAE (Fig. 3, *A–C*, and Table II) strongly support this notion.

So far, several types of T cells involved in the negative regulation of immune responses have been identified, such as IL-10-producing Tr1 cells and $CD4^+CD25^+$ Treg (17, 19, 23). Kohm et al. (24) reported that the adoptive transfer of relatively large number of $CD4^+CD25^+$ Treg (2×10^6) isolated from naive mice protected the recipient mice from MOG-induced EAE. It was recently reported that immature DC induced Tr1 cells producing high amounts of IL-10 (25), and also that the proliferation of $CD4^+CD25^+$ Treg was efficiently promoted by DC (17). We thus attempted to characterize the T cells with regulatory activity induced by ES-DC expressing TRAIL and involved in the protection from EAE. We quantified IL-10, IFN-γ, and IL-4 produced by spleen cells isolated from ES-DC-treated mice upon in vitro stimulation with MOG peptide, by ELISA. No significant change in the amount of these cytokines produced by spleen cells of ES-DC-TRAIL/MOG, ES-DC-PDL1/MOG, or ES-DC-MOG-treated mice was observed (data not shown). We thus considered it less likely that the disease-prevention effect was mediated by IL-10-producing Tr1 cells or Th2 cells, although we cannot totally rule out this possibility.

We next assessed the possibility that ES-DC-TRAIL/MOG had propagated or activated $CD4^+CD25^+$ Treg in vivo. Adoptive transfer experiments showed the presence of $CD4^+CD25^+$ T cells with a capacity to prevent not only MOG- but also MBP-induced EAE in ES-DC-TRAIL/MOG-treated mice (Fig. 3, *D–F*, and Table II). In addition, when $CD4^+CD25^+$ T cells were depleted by the pretreatment of mice with anti-CD25 mAb, the protective effect of ES-DC-TRAIL/MOG against MBP-induced EAE was totally abrogated (Fig. 4 and Table I), the observation further supporting the notion that $CD4^+CD25^+$ T cells play a role in the prevention of MBP-induced EAE by treatment with ES-DC-TRAIL/MOG. Recently, Kohm et al. (26) reported that the effect of anti-CD25 Ab is not the depletion but functional inactivation of Treg. Our findings that preventive effect of MBP-induced EAE with ES-DC-TRAIL/MOG was diminished by the injection of anti-CD25 mAb into mice also may be interpreted as inactivation of Treg. Several groups recently reported that $CD4^+CD25^-$ T cells converted to Treg functionality in particular condition (27, 28). It is possible that $CD4^+CD25^+$ Tregs suppressing EAE observed in our study were also those converted from conventional $CD4^+$ T cells.

Furthermore, we observed an increased number of $Foxp3^+$ cells and the ratio of $Foxp3^+$ cells to $CD4^+$ cells in the spinal cords in mice treated with ES-DC-TRAIL/MOG (Fig. 5). In contrast, we could not detect any significant increase in the expression of Foxp3 mRNA in the spleen of ES-DC-TRAIL/MOG-treated mice (data not shown). Also in flow cytometry analysis the proportion of $Foxp3^+$ cells to $CD4^+$ cells in the spleen and inguinal lymph nodes was not increased in mice treated with ES-DC-TRAIL/MOG as compared with control mice (the proportion of $Foxp3^+$ cells to $CD4^+$ cells was 9.7 ± 0.7% in spleen of control mice, 9.8 ± 0.8% in spleen of ES-DC-TRAIL/MOG-treated mice, 9.2 ± 0.9% in inguinal lymph nodes of control mice, and 9.5 ± 0.1% in inguinal lymph nodes of ES-DC-TRAIL/MOG-treated mice). Probably the increase in the number of MOG-reactive $Foxp3^+$ cells was too small to be detected in total $CD4^+$ T cells of spleen and inguinal lymph nodes. In addition, to investigate whether the ES-DC-TRAIL/MOG were acting at the level of priming or within the target organ (CNS), we tested the primary proliferative response to MBP of the T cells of mice treated with ES-DC-TRAIL/MOG before immunization with MBP for EAE induction (as shown in Fig. 1*A*). The inguinal lymph node cells were harvested on day 19 after the immunization and cultured in the presence of MBP (whole protein, 0, 12.5, 25, 50 μg/ml) for 3 days. In this experiment, the treatment with ES-DC-TRAIL/MOG did not reduce the proliferative response to MBP of T cells in inguinal lymph node (data not shown). This result suggests that treatment with ES-DC-TRAIL/MOG did not act at the level of priming. It may be more likely that the treatment with ES-DC-TRAIL/MOG acted in the target organ (CNS), as shown in Fig. 7.

The $CD4^+CD25^+$ T cells induced by ES-DC-TRAIL/MOG and responsible for disease prevention were probably specific to MOG, because ES-DC expressing TRAIL along with irrelevant Ag (OVA) had no effect on MBP-induced EAE (Figs. 1*D* and 3*F* and Tables I and II).

Consistent with recent reports on the stimulation of $CD4^+CD25^+$ Treg as well as conventional T cells by splenic or BM-derived DC (17, 29), in vitro experiments showed that ES-DC also have the capacity to induce proliferation of both $CD4^+CD25^-$ conventional T cells and $CD4^+CD25^+$ Treg, when stimulated with anti-CD3 mAb in the presence of low-dose IL-2. The expression of TRAIL on ES-DC enhanced the capacity to induce proliferation of $CD4^+CD25^+$ Treg, but not of $CD4^+CD25^-$ conventional T cells (Fig. 6, *A* and *B*). In addition, anti-TRAIL-blocking mAb decreased the proliferation of

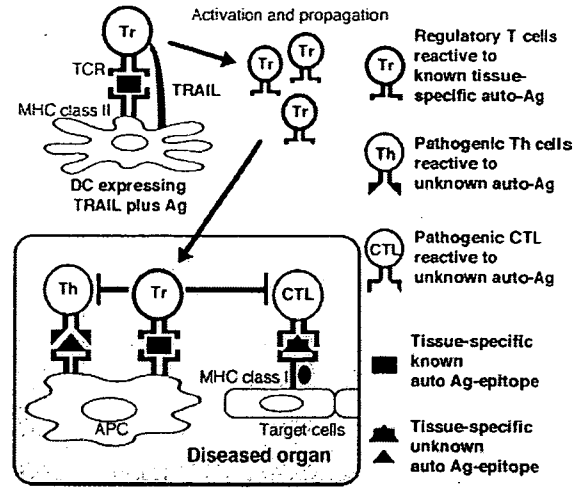


FIGURE 7. Promotion of Tregs with DC expressing TRAIL plus certain tissue-specific autoantigen as a novel strategy for the treatment of the organ-specific autoimmune diseases. DC expressing TRAIL and simultaneously presenting known tissue-specific autoantigen (auto-Ag) in the context of their MHC class II can activate or propagate T cells with a regulatory function (Tr cells) reactive to the autoantigen. In the focus of autoimmune disease, the promoted-Tr cells were retriggered by the same autoantigen expressed in the tissue. Then, the Tr cells inhibited the pathogenic T cells responding to unknown autoantigen. This strategy is applicable, even if target autoantigens are unidentified or if multiple Ags are recognized as targets by the pathogenic autoreactive T cells.

Treg cocultured with ES-DC-TRAIL or natural APC, such as LPS-treated spleen cells or macrophage (Fig. 6, *C–F*). The results of these in vitro experiments suggest that the in vivo transfer of ES-DC-TRAIL/MOG induced proliferation of MOG-reactive Treg which protected the recipient mice from EAE.

Based on the results obtained in the current study, we consider that the inhibition of autoimmunity by TRAIL-expressing ES-DC may be attributed to the promotion of $CD4^+CD25^+$ T cells by TRAIL, in addition to the induction of apoptosis of pathogenic T cells as suggested by our previous study (1). Mi et al. (30) reported that TRAIL inhibited the proliferation of diabetogenic T cells isolated from NOD mice by suppressing IL-2 production and upregulating the expression of $p27^{kip1}$. It may be possible that the effects of Treg were involved in their observations. In addition, Herbeuval et al. (31) reported that a level of TRAIL was elevated in plasma of HIV-1-infected patients and in vitro exposure to HIV-1 induced the expression of TRAIL in APCs. Andersson et al. (32) reported expression of Foxp3 to be enhanced in the lymphoid tissue of HIV-infected patients. These two findings may also be related to our findings.

DC modified by some way to enhance their tolerogenic characteristics is regarded as a promising therapeutic means to negatively manipulate the immune response for the treatment of autoimmune and allergic diseases and also for the induction of transplantation tolerance (1, 23, 33–36). In the clinically manifest phase of autoimmune diseases, such as multiple sclerosis or type I diabetes, it is presumed that multiple tissue-specific Ags are recognized as targets by deregulated immunity due to epitope spreading (37). As a result, the induction of a mere deletion or anergy of pathogenic T cells specific to primarily recognized autoantigens may not be sufficient to control these diseases. The promotion of the immune-suppressive T cells reactive to organ-specific self Ags by treatment with genetically modified DC may be a promising therapeutic modality for subjects with autoimmune diseases (Fig. 7). This strategy may also be useful for the induction of transplantation tolerance.

Acknowledgments

We thank Dr. S. Aizawa (RIKEN Center for Developmental Biology, Kobe, Japan) for TT2, Drs. N. Takakura (Kanazawa University, Kanazawa, Japan) and T. Suda (Keio University, Tokyo, Japan) for OP9, Dr. H. Niwa (RIKEN Center for Developmental Biology, Kobe, Japan) for pCAG-IPuro, Drs. T. Okazaki and T. Honjo (Kyoto University, Kyoto, Japan) for a cDNA clone for PD-L1, and Drs. T. Koda and T. Nishimura (Hokkaido University, Sapporo, Japan) for a cDNA clone for OVA.

Disclosures

The authors have no financial conflict of interest.

References

1. Hirata, S., S. Senju, H. Matsuyoshi, D. Fukuma, Y. Uemura, and Y. Nishimura. 2005. 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.* 174: 1888–1897.
2. Wiley, S. R., K. Schooley, P. J. Smolak, W. S. Din, C. P. Huang, J. K. Nicholl, G. R. Sutherland, T. D. Smith, C. Rauch, C. A. Smith, et al. 1995. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* 3: 673–682.
3. Kayagaki, N., N. Yamaguchi, M. Nakayama, K. Takeda, H. Akiba, H. Tsutsui, H. Okamura, K. Nakanishi, K. Okumura, and H. Yagita. 1999. Expression and function of TNF-related apoptosis-inducing ligand on murine activated NK cells. *J. Immunol.* 163: 1906–1913.
4. Nieda, M., A. Nicol, Y. Koezuka, A. Kikuchi, N. Lapteva, Y. Tanaka, K. Tokunaga, K. Suzuki, N. Kayagaki, H. Yagita, et al. 2001. TRAIL expression by activated human $CD4^+V\alpha24$NKT cells induces in vitro and in vivo apoptosis of human acute myeloid leukemia cells. *Blood* 97: 2067–2074.
5. Raftery, M. J., M. Schwab, S. M. Eibert, Y. Samstag, H. Walczak, and G. Schonrich. 2001. Targeting the function of mature dendritic cells by human cytomegalovirus: a multilayered viral defense strategy. *Immunity* 15: 997–1009.
6. Hilliard, B., A. Wilmen, C. Seidel, T. S. Liu, R. Goke, and Y. Chen. 2001. Roles of TNF-related apoptosis-inducing ligand in experimental autoimmune encephalomyelitis. *J. Immunol.* 166: 1314–1319.
7. Lamhamedi-Cherradi, S. E., S. J. Zheng, K. A. Maguschak, J. Peschon, and Y. H. Chen. 2003. Defective thymocyte apoptosis and accelerated autoimmune diseases in $TRAIL^{-/-}$ mice. *Nat. Immunol.* 4: 255–260.
8. Song, K., Y. Chen, R. Goke, A. Wilmen, C. Seidel, A. Goke, and B. Hilliard. 2000. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is an inhibitor of autoimmune inflammation and cell cycle progression. *J. Exp. Med.* 191: 1095–1104.
9. Lunemann, J. D., S. Waiczies, S. Ehrlich, U. Wendling, B. Seeger, T. Kamradt, and F. Zipp. 2002. Death ligand TRAIL induces no apoptosis but inhibits activation of human (auto)antigen-specific T cells. *J. Immunol.* 168: 4881–4888.
10. Senju, S., S. Hirata, H. Matsuyoshi, M. Masuda, Y. Uemura, K. Araki, K. Yamamura, and Y. Nishimura. 2003. Generation and genetic modification of dendritic cells derived from mouse embryonic stem cells. *Blood* 101: 3501–3508.
11. Matsuyoshi, H., S. Senju, S. Hirata, Y. Yoshitake, Y. Uemura, and Y. Nishimura. 2004. 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.* 172: 776–786.
12. Motomura, Y., S. Senju, T. Nakatsura, H. Matsuyoshi, S. Hirata, M. Monji, H. Komori, D. Fukuma, H. Baba, and Y. Nishimura. 2006. Embryonic stem cell-derived dendritic cells expressing glypican-3, a recently identified oncofetal antigen, induce protective immunity against highly metastatic mouse melanoma, B16–F10. *Cancer Res.* 66: 2414–2422.
13. Fukuma, D., H. Matsuyoshi, S. Hirata, A. Kurisaki, Y. Motomura, Y. Yoshitake, M. Shinohara, Y. Nishimura, and S. Senju. 2005. Cancer prevention with semi-allogeneic ES cell-derived dendritic cells. *Biochem. Biophys. Res. Commun.* 335: 5–13.
14. Fujii, S., S. Senju, Y. Z. Chen, M. Ando, S. Matsushita, and Y. Nishimura. 1998. The CLIP-substituted invariant chain efficiently targets an antigenic peptide to HLA class II pathway in L cells. *Hum. Immunol.* 59: 607–614.
15. Uemura, Y., S. Senju, K. Maenaka, L. K. Iwai, S. Fujii, H. Tabata, H. Tsukamoto, S. Hirata, Y. Z. Chen, and Y. Nishimura. 2003. 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: 947–960.
16. Morgan, M. E., R. P. Sutmuller, H. J. Witteveen, L. M. van Duivenvoorde, E. Zanelli, C. J. Melief, A. Snijders, R. Offringa, R. R. de Vries, and R. E. Toes. 2003. $CD25^+$ cell depletion hastens the onset of severe disease in collagen-induced arthritis. *Arthritis Rheum.* 48: 1452–1460.
17. Yamazaki, S., T. Iyoda, K. Tarbell, K. Olson, K. Velinzon, K. Inaba, and R. M. Steinman. 2003. Direct expansion of functional $CD25^+CD4^+$ regulatory T cells by antigen-processing dendritic cells. *J. Exp. Med.* 198: 235–247.
18. Sakaguchi, S. 2003. The origin of FOXP3-expressing $CD4^+$ regulatory T cells: thymus or periphery. *J. Clin. Invest.* 112: 1310–1312.
19. Walker, L. S., A. Chodos, M. Eggena, H. Dooms, and A. K. Abbas. 2003. Antigen-dependent proliferation of $CD4^+CD25^+$ regulatory T cells in vivo. *J. Exp. Med.* 198: 249–254.
20. Mariani, S. M., and P. H. Krammer. 1998. Surface expression of TRAIL/Apo-2 ligand in activated mouse T and B cells. *Eur. J. Immunol.* 28: 1492–1498.
21. Santini, S. M., C. Lapenta, M. Logozzi, S. Parlato, M. Spada, T. Di Pucchio, and F. Belardelli. 2000. Type I interferon as a powerful adjuvant for monocyte-derived dendritic cell development and activity in vitro and in Hu-PBL-SCID mice. *J. Exp. Med.* 191: 1777–1788.
22. Yu, Y., S. Liu, W. Wang, W. Song, M. Zhang, W. Zhang, Z. Qin, and X. Cao. 2002. Involvement of tumour necrosis factor-α-related apoptosis-inducing ligand in enhanced cytotoxicity of lipopolysaccharide-stimulated dendritic cells to activated T cells. *Immunology* 106: 308–315.
23. Menges, M., S. Rossner, C. Voigtlander, H. Schindler, N. A. Kukutsch, C. Bogdan, K. Erb, G. Schuler, and M. B. Lutz. 2002. Repetitive injections of dendritic cells matured with tumor necrosis factor α induce antigen-specific protection of mice from autoimmunity. *J. Exp. Med.* 195: 15–21.
24. Kohm, A. P., P. A. Carpentier, H. A. Anger, and S. D. Miller. 2002. Cutting edge: $CD4^+CD25^+$ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis. *J. Immunol.* 169: 4712–4716.
25. Wakkach, A., N. Fournier, V. Brun, J. P. Breittmayer, F. Cottrez, and H. Groux. 2003. Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. *Immunity* 18: 605–617.
26. Kohm, A. P., J. S. McMahon, J. R. Podojil, W. S. Begolka, M. DeGutes, D. J. Kasprowicz, S. F. Ziegler, and S. D. Miller. 2006. Cutting edge: anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of $CD4^+CD25^+$ T regulatory cells. *J. Immunol* 176: 3301–3305.
27. Liang, S., P. Alard, Y. Zhao, S. Parnell, S. L. Clark, and M. M. Kosiewicz. 2005. Conversion of $CD4^+CD25^-$cells into $CD4^+CD25^+$ regulatory T cells in vivo requires B7 costimulation, but not the thymus. *J. Exp. Med.* 201: 127–137.
28. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral $CD4^+CD25^-$naive T cells to $CD4^+CD25^+$ regulatory T cells by TGF-β induction of transcription factor Foxp3. *J. Exp. Med.* 198: 1875–1886.
29. Tarbell, K. V., S. Yamazaki, K. Olson, P. Toy, and R. M. Steinman. 2004. $CD25^+CD4^+$ T cells, expanded with dendritic cells presenting a single autoantigenic peptide, suppress autoimmune diabetes. *J. Exp. Med.* 199: 1467–1477.
30. Mi, Q. S., D. Ly, S. E. Lamhamedi-Cherradi, K. V. Salojin, L. Zhou, M. Grattan, C. Meagher, P. Zucker, Y. H. Chen, J. Nagle, et al. 2003. Blockade of tumor necrosis factor-related apoptosis-inducing ligand exacerbates type 1 diabetes in NOD mice. *Diabetes* 52: 1967–1975.
31. Herbeuval, J. P., A. Boasso, J. C. Grivel, A. W. Hardy, S. A. Anderson, M. J. Dolan, C. Chougnet, J. D. Lifson, and G. M. Shearer. 2005. TNF-related apoptosis-inducing ligand (TRAIL) in HIV-1-infected patients and its in vitro production by antigen-presenting cells. *Blood* 105: 2458–2464.
32. Andersson, J., A. Boasso, J. Nilsson, R. Zhang, N. J. Shire, S. Lindback, G. M. Shearer, and C. A. Chougnet. 2005. Cutting edge: the prevalence of regulatory T cells in lymphoid tissue is correlated with viral load in HIV-infected patients. *J. Immunol.* 174: 3143–3147.
33. Lu, L., and A. W. Thomson. 2002. Manipulation of dendritic cells for tolerance induction in transplantation and autoimmune disease. *Transplantation* 73: S19–S22.
34. Liu, Z., X. Xu, H. C. Hsu, A. Tousson, P. A. Yang, Q. Wu, C. Liu, S. Yu, H. G. Zhang, and J. D. Mountz. 2003. CII-DC-AdTRAIL cell gene therapy inhibits infiltration of CII-reactive T cells and CII-induced arthritis. *J. Clin. Invest.* 112: 1332–1341.
35. Terness, P., T. M. Bauer, L. Rose, C. Dufter, A. Watzlik, H. Simon, and G. Opelz. 2002. Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. *J. Exp. Med.* 196: 447–457.
36. Sato, K., T. Nakaoka, N. Yamashita, H. Yagita, H. Kawasaki, C. Morimoto, M. Baba, and T. Matsuyama. 2005. TRAIL-transduced dendritic cells protect mice from acute graft-versus-host disease and leukemia relapse. *J. Immunol.* 174: 4025–4033.
37. Vanderlugt, C. L., and S. D. Miller. 2002. Epitope spreading in immune-mediated diseases: implications for immunotherapy. *Nat. Rev. Immunol.* 2: 85–95.

ORIGINAL ARTICLE

The novel *IFNGR1* mutation 774del4 produces a truncated form of interferon-γ receptor 1 and has a dominant-negative effect on interferon-γ signal transduction

Satoshi Okada, Nobutsune Ishikawa, Ken'ichiro Shirao, Hiroshi Kawaguchi, Miyuki Tsumura, Yoshinori Ohno, Shin'ichiro Yasunaga, Motoaki Ohtsubo, Yoshihiro Takihara, Masao Kobayashi

J Med Genet 2007;**44**:485–491. doi: 10.1136/jmg.2007.049635

See end of article for authors' affiliations

Correspondence to: Masao Kobayashi, MD, Department of Pediatrics, Hiroshima University Graduate School of Biomedical Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan; masak@hiroshima-u.ac.jp

Received 8 February 2007
Revised 17 April 2007
Accepted 26 April 2007
Published Online First 18 May 2007

Background: Patients with interferon-γ receptor 1 (IFNγR1) deficiency show selective susceptibility to intracellular pathogens such as mycobacteria. IFNγR1 deficiency is an inherited immunodeficiency disorder, which can be either recessive or dominant. Dominant forms of IFNγR1 deficiency are known to be associated with mutations that introduce a premature stop codon in the intracellular domain of IFNγR1. One such mutation, 818del4, is believed to be the most common type. Although these mutations are presumed to exert a dominant-negative effect on IFNγ signal transduction, the underlying molecular mechanism is unresolved.
Objective: We characterised the 774del4 mutant of IFNγR1 using a gene-expression system to examine the effects of this mutation on IFNγ signal transduction.
Results: We identified a novel dominant mutation in *IFNGR1*, designated 774del4, which produced a truncated form of IFNγR1 in a patient with recurrent mycobacterial infections. IFNγR1 was overexpressed on the surfaces of CD14-positive cells from the peripheral blood of this patient, and STAT1 phosphorylation in response to high doses of IFNγ was partially deficient. We expressed two truncated forms of IFNγR1, 774del4 and 818del4, in HEK 293 cells using transient transfection and found that these mutants overexpressed IFNγR1 on the cell surface because of impaired receptor stability, which resulted in a dominant-negative effect on IFNγ signal transduction.
Conclusion: Like the 818del4 mutation, 774del4 produces a truncated form of IFNγR1, which has a dominant-negative effect on IFNγ signal transduction through altered receptor stability.

Mendelian susceptibility to mycobacterial diseases (MSMD; OMIM 209950) is a rare congenital disorder characterised by susceptibility to infection by poorly virulent intracellular pathogens such as bacillus Calmette-Guérin (BCG) and non-tuberculosis mycobacterium (NTM), without any other marked immunodeficiency.[1] MSMD is caused by an underlying genetic defect in the IL-12/23–IFNγ circuit that enhances the pathogenesis of BCG and NTM. To date, six genes (*IFNGR1, IFNGR2, IL12B, IL12RB1, STAT1*, and *NEMO*) are known to be associated with this disorder.[2–11] Interferon-γ receptor 1 (IFNγR1) deficiency, caused by mutations in *IFNGR1*, is included in this disease category and is clinically characterised by disseminated BCG and severe NTM infections. This inherited immunodeficiency exists in both recessive and dominant forms, with a high degree of allelic heterogeneity.[2–4] The dominant forms of IFNγR1 deficiency are frequently associated with a mutation in the intracellular domain of *IFNGR1*; in particular, the mutant known as 818del4 involves a 4 bp deletion in exon 6. In addition, the 811del4, 817insA, 818delT and G832T mutations have been reported.[12–15] In this study, we identified a novel dominant mutation in *IFNGR1*, designated 774del4, in a patient with multifocal osteomyelitis due to mycobacterial infection. Although these mutations are presumed to exert a dominant-negative effect on IFNγ signal transduction, the exact molecular mechanism is unknown. Therefore, we used a gene-expression system in heterologous cells to examine the effects of the mutant receptors on IFNγ signalling. We found that the truncated forms of IFNγR1 are overexpressed on the cell surface because of altered receptor stability, resulting in a dominant-negative effect on IFNγ signal transduction.

MATERIALS AND METHODS

Case report

The subject was a Japanese girl who was vaccinated with BCG at 4 months of age. She developed regional lymph node enlargement 2 months after vaccination, and lymph-node biopsies revealed a tuberculoid granuloma. She was diagnosed with BCG lymphadenitis and treated with isoniazid for 3 years. At 12 years of age, she developed pain in her left knee and back, and persistent low-grade fever. Radiography indicated multifocal osteomyelitis. Bone biopsies revealed tuberculoid granulomas, and PCR detected the presence of *Mycobacterium avium*. The patient was treated with isoniazid, rifampicin, and clarithromycin or azithromycin, and her symptoms gradually improved.

We obtained blood samples from the patient, her relatives and healthy adult controls, after obtaining informed consent. This study was approved by the ethics committee and internal review board of Hiroshima University.

Molecular genetics

Genomic DNA was extracted from peripheral blood leucocytes. Exon 6 of *IFNGR1* and its flanking introns were amplified by PCR using the following primers: sense 5′-GTGTTCTTTAA AACTCTGGCC-3′ and antisense 5′-TGGTAGACTGACTGATTG

Abbreviations: APC, antigen-presenting cell; BCG, bacillus Calmette-Guérin; FCS, fetal calf serum; IFNγR1, interferon gamma receptor 1; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; MSMD, mendelian susceptibility to mycobacterial diseases; NTM, non-tuberculosis mycobacterium; PBMC, peripheral blood mononuclear cell; STAT1, signal transducer and activator of transcription 1; TNF, tumour necrosis factor; WT, wild type

ATG-3'. The products were sequenced directly and then further analysed by cycle sequencing (BigDye Terminator v3.1 cycle sequencing kit; Applied Biosystems, Foster City, California, USA) and run on an automated analyser (ABI PRISM 310 Genetic Analyzer; Applied Biosystems).

Total RNA was extracted from the peripheral blood mononuclear cells (PBMCs) using Isogen extraction (Nippon Gene Co., Tokyo, Japan), and cDNA was synthesised from 5 μg of total RNA using a first-strand synthesis system for RT-PCR (SuperScript; Invitrogen, Carlsbad, California, USA). PCR of the wild-type (WT) and 774del4 mutant alleles was performed with primers that span the entire coding region of IFNγR1 (5'-GGATCCCGCAGGCGCTCGGGGTTGGA-3' and 5'-CTCGAGGAAAACTGTCCAGGAAAATCAGACT-3'). The PCR products were cloned into pGEM-T Easy vector (Promega, Madison, Wisonsin, USA). To generate the 818del4 mutant, we performed PCR-based mutagenesis of the WT construct using the following mismatched PCR primers: sense 5'-TTTATATTAAGAAAATCCATTGAAGGAAAA-3' and antisense, 5'-TTTTCCTTCAATGGATTTTCTTAATATAAA-3'. These fragments were subcloned into the *Bam*HI and *Xho*I sites of a mammalian expression vector pcDNA (Invitrogen).

Flow cytometry

PBMCs were stimulated with 20 μg/L lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, Missouri, UK) and 100 μg/L macrophage colony-stimulating factor (M-CSF) (Kyowa Hakko Kogyo, Tokyo, Japan) for 48 h. Portions of the cells were stained with phycoerythrin-conjugated monoclonal anti-human IFNγR1 antibody (GIR-94; Santa Cruz Biotechnology, Santa Cruz, CA) or the isotype control, and analysed by gating in a FACS Calibur apparatus (Becton Dickinson, Franklin Lakes, New Jersey, USA). The remaining cells were recultured for an additional 18 h under starvation conditions and then left untreated, or treated with 50 μg/L LPS and 10 000 U/mL IFNγ for 30 min at 37℃. After stimulation, the cells were analysed by flow cytometry using an anti-Stat1 antibody (BD Phosflow anti-Stat1 (pY701); Becton Dickinson).

Analysis of gene expression

The HEK 293 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS) (HyClone, Logan, Utah, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. At 24 h before transfection, the cells were harvested by trypsinisation and replated at a density of 1×10^5 cells/mL in 100 mm culture dishes. Plasmid DNA (10 μg per plate) carrying the WT, 774del4 or 818del4 alleles of *IFNGR1* was introduced into the HEK 293 cells by calcium phosphate-mediated transfection.

At 24 h after transfection, a portion of the cells was used for Western blotting[16] to detect the IFNγR1 protein using anti-human IFNγR1 antibodies. The remaining cells were treated with 20 μg/ml cycloheximide for 3 h and analysed by flow cytometry and Western blotting.

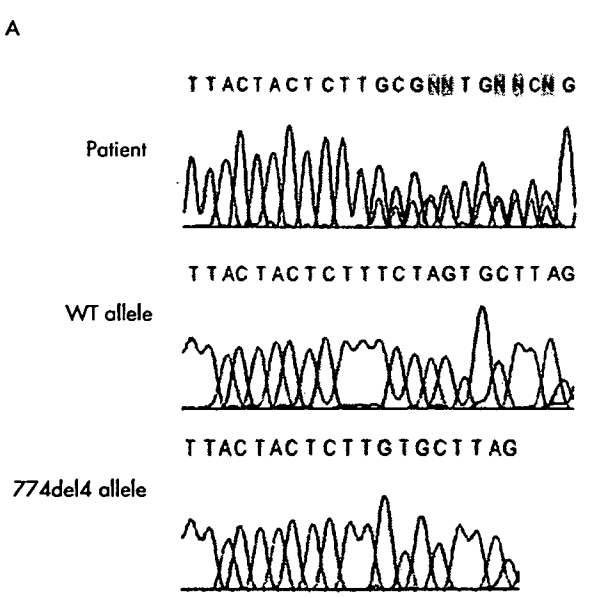
Primary cell separation and cytokine measurements

CD14-positive cells and CD3-positive cells were purified from PBMCs using a cell separation system (IMag; Becton-Dickinson) according to the manufacturer's instructions. The purity of the CD14-positive cell population was >90%, and that of the CD3-positive cell population was >97% (data not shown). The CD14-positive cells were cultured for 48 h in RPMI 1640 (Sigma-Aldrich) supplemented with 15% FCS, with the addition of 500 ng/mL LPS and various concentrations (10^2–10^5 IU/mL) of IFNγ (Genentech, South San Francisco, California, USA). The cell-culture supernatants were harvested, and the concentration of tumour necrosis factor (TNF)-α was measured in duplicate using a human TNFα antibody bead kit (Biosource International, Camarillo, California, USA) and Luminex system (Luminex, Austin, Texas, USA). The CD3-positive cells were incubated for 48 h with 1 μg/mL phytohaemagglutinin (PHA) (Sigma-Aldrich) and various concentrations (1–10^5 pg/mL) of IL-12. The concentration of IFNγ was measured as described above using Luminex.

RESULTS

Sequence analysis

High molecular weight DNA was extracted from the peripheral blood samples, and the exons and flanking introns of *IFNGR1* were amplified by PCR. Sequence analysis revealed that the patient had a heterozygous mutation, 774del4 (TCTA), in exon 6 of *IFNGR1* (figure 1A). This mutation was not detected in the subject's parents or in 30 healthy Japanese controls (figure 1B).



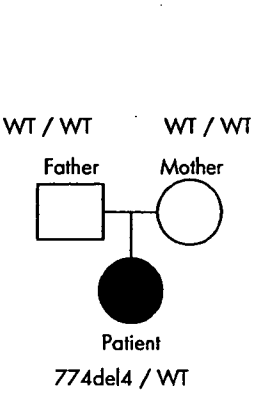


Figure 1 Sequence analysis of exon 6 of *IFNGR1*. (A) The genomic DNA of *IFNGR1* exon 6 from the patient was amplified by PCR, and the sequence was analysed by direct sequencing. The PCR product was cloned into the pGEM-T Easy vector. Sequencing analysis detected the presence of the wild-type (WT) and 774del4 alleles and that the patient had a heterozygous mutation, 774del4 (TCTA), in exon 6 of *IFNGR1*. (B) Pedigree of this family with the dominant form of IFNγR1 deficiency. Filled symbol, affected person; open symbols, healthy family member. The 774del4 mutation was not detected in either of the parents.

Response to IFNγ

To ascertain the cellular response to IFNγ, we purified CD14-positive cells from the PBMCs of the patient and controls. The CD14-positive cells from the patient produced a lower level of TNFα in response to IFNγ stimulation, regardless of the dose (figure 2A). Once activated, the IFNγ receptor induces STAT1 homodimerisation via phosphorylation of tyrosine residue 701, and these homodimers enter the nucleus, where they act as transcription factors. Therefore, STAT1 Tyr^{701} phosphorylation (p-Y701-STAT1) in response to IFNγ was examined in PBMCs from the patient. PBMCs were incubated with M-CSF and LPS for 48 h and then recultured for an additional 18 h under starvation conditions. The cells were then treated with or without LPS and IFNγ for 30 min or left untreated, and the level of p-Y701-STAT1 was analysed by flow cytometry. Although STAT1 phosphorylation was detected in the patient, it was significantly reduced (figure 3B).

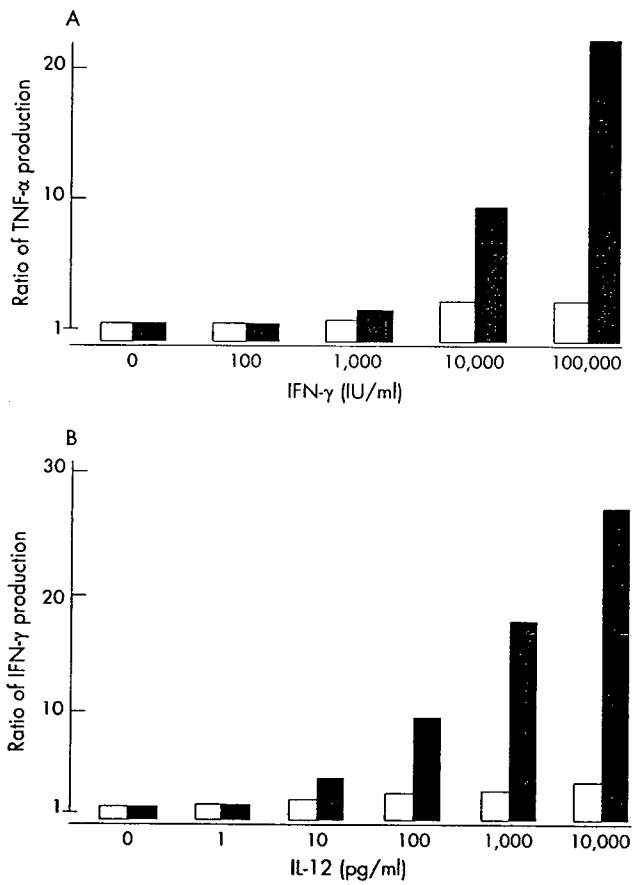


Figure 2 Analysis of cytokine production. (A) CD14-positive mononuclear cells in the peripheral blood samples of a patient and normal controls were stimulated with LPS and various concentrations of interferon (IFN)-γ for 48 hours. The purity levels of the CD14-positive cell populations were >90%. Cell-culture supernatants were harvested, and the concentrations of tumour necrosis factor (TNF)-α were measured by Luminex. The data represent the ratios of the levels of TNFα produced in response to LPS plus IFNγ to the levels of TNFα produced in response to LPS alone. Open columns, data from the patient; filled column, representative data from control subjects. (B) CD3-positive mononuclear cells in the peripheral blood samples of a patient and normal controls were stimulated with purified hemagglutinin (PHA) 1 μg/ml and various concentrations of interleukin (IL)-12 for 48 hours. The purity levels of CD3-positive cells populations were >95%. Cell-culture supernatants were harvested, and the concentrations of IFNγ were measured by Luminex. The data represent the ratios of IFNγ produced in response to PHA plus IL-12 to the levels of IFNγ produced in response to PHA alone. Open columns, data from the patient; filled columns, representative data from the control subjects.

Expression of the IFNγR1 mutant proteins

The 774del4 mutation was presumed to cause a frameshift that would lead to a premature stop codon at nucleotide positions 827–829, thereby producing a truncation in the cytoplasmic domain of the receptor.[4 17–19] To characterise the 774del4 mutant protein, PBMCs from the patient and healthy controls were isolated and stimulated with LPS and M-CSF for 48 h, and surface expression of IFNγR1 on the activated CD14-positive mononuclear cells was analysed using flow cytometry. Increased expression of IFNγR1 was detected in the patient (figure 3A).

To characterise the 774del4 and 818del4 mutant versions of IFNγR1 further, we cloned the cDNAs for these mutants into pcDNA and transiently transfected each construct into HEK 293 cells, using calcium phosphate-mediated transfection. The gene products were analysed by Western blot analysis (figure 4). The 774del4 and 818del4 mutant proteins had apparent molecular weights in the range of 45–60 kDa, in contrast to the WT molecular weight of 80–95 kDa. These results indicate that the 774del4 and 818del4 mutant alleles of *IFNGR1* encode truncated forms of IFNγR1 that accumulate on the cell surface, as predicted by the primary structures of the mutants.

IFNγR1 stability

As cell-surface overexpression of the 774del4 truncated form of IFNγR1 was detected in the patient, we established HEK 293 cells that transiently expressed either 774del4 or 818del4 and examined the stability of the truncated proteins. HEK 293 cells transiently transfected with either of the vectors were treated with cycloheximide for 3 h, and IFNγR1 expression was analysed by Western blotting (figure 5B) and flow cytometry (figure 5A). The 774del4 and 818del4 mutant versions of IFNγR1 were consistently detected, even after the inhibition of translation by cycloheximide (see supplementary figure, available online at http://jmg.bmj.com/supplemental). This result indicates that these mutant proteins are stably expressed on the cell surface due to alteration of receptor stability.

Cellular response to IL-12

To examine the cellular response to IL-12, we purified CD3-positive cells from the PBMCs of the patient and the controls. Compared with the CD3-positive cells of the controls, those of the patient produced very little IFNγ in response to IL-12 (figure 2B).

DISCUSSION

IFNγ and IL-12 play important roles in initiating and sustaining the Th1 cytokine cascade. Antigen-presenting cells (APCs), including macrophages and dendritic cells, produce IL-12 in response to mycobacterial infection. IL-12 stimulates T cells and natural killer cells through its receptor (a heterodimer of IL-12 receptors β1 and β2) to produce IFNγ. IFNγ acts through its receptor (a heterodimer of IFNγR1 and IFNγR2) on macrophages to activate the defence against mycobacteria. IFNγ signalling in APCs increases microbiocidal activity and upregulates IL-12 and TNFα production through STAT1 activation. Thus, IFNγ and IL-12 work synergistically to induce cellular immunity,[20 21] and impairment of the IL-12/23-IFNγ circuit results in MSMD. Recently, mutations were identified in the *IFNGR1*, *IFNGR2*, *IL12B*, *IL12RB1*, *STAT1*, and *NEMO* genes of patients with MSMD.[2–11] In fact, 13 different genetic disorders associated with MSMD have been shown to be caused by mutations in these six genes.[11] In particular, defects in *IFNGR1*, *IFNGR2*, and *STAT1* are associated with impaired cellular responses to IFNγ, and defects in *IL12B*, *IL12RB1*, and *NEMO* are associated with IL-12-dependent IFNγ production.[11] Further, an X-linked recessive form of MSMD was recently

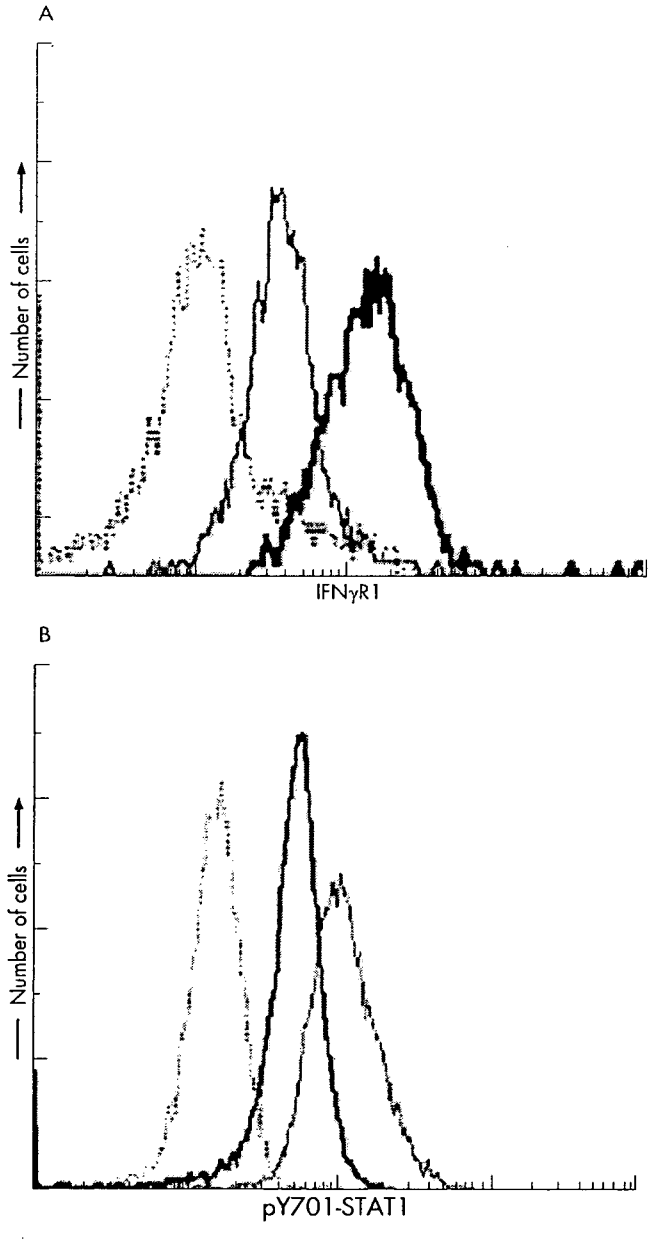


Figure 3 Analysis of cell surface IFNγR1 and STAT1 phosphorylation in response to IFNγ stimulation. (A) Peripheral blood mononuclear cells were cultured with RPMI 1640 containing 15% FCS, 50 μg/l LPS and 100 μg/l M-CSF for 48 hours. The cells were stained with phycoerythrin (PE)-conjugated anti-interferon γ receptor 1 (IFNγR1) antibody and fluorescein isothiocyanate (FITC)-conjugated anti-CD14 antibody and analysed by flow cytometry. The data represent the levels of expression of IFNγR1 on CD14-positive cells. Bold line, patient; solid line, control. The dotted line indicates binding of the appropriate isotype control antibody. (B) Peripheral blood mononuclear leukocytes were incubated in RPMI 1640 that was supplemented with 15% fetal calf serum (FCS), 100 μg/l macrophage colony-stimulating factor (M-CSF), and 20 μg/l lipopoly-saccharide (LPS) for 48 hours. The harvested cells were washed and then cultured for an additional 18 hours in RPMI 1640 medium alone. Then cells were treated with or without 50 μg/l LPS and 10 000 U/ml IFNγ for 30 minutes, and the cells were then stained with anti-STAT1 (pY701) antibody and analysed by flow cytometry. Bold line, patient sample with cytokine stimulation; solid line, normal subject sample with cytokine stimulation; dotted line, normal subject sample without cytokine stimulation.

identified and has been mapped to two candidate regions, Xp11.4-Xp21.2 and Xq25-Xq26.3.[22] Patients bearing these mutant alleles share certain clinical features, including selective susceptibility to mycobacteria, *Salmonellae* and some viral

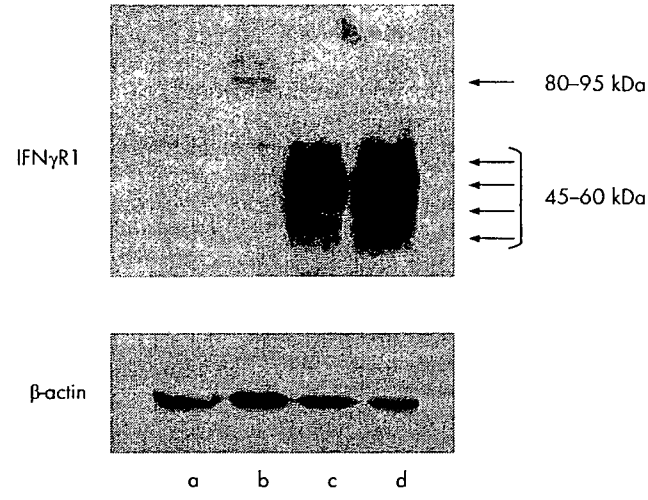


Figure 4 Expression of 774del4 and 818del4 mutant IFNγR1 proteins by transfected cells. Expression constructs carrying the wild-type (WT), 774del4, and 818del4 forms of *IFNGR1* were transiently transfected into HEK 293 cells using the calcium phosphate method. IFNγR1 protein was detected by Western blotting. The WT IFNγR1 (B) was detected with an apparent molecular weight of 80–95 kDa. The 774del4 (C) and 818del4 (D) mutant forms of IFNγR1 gave strong signals with an apparent molecular weight of 45–60 kDa; this band was not detected in the WT-transfected cell lysate. IFNγR1 was not detected in the total lysate of cells transfected with the mock vector (A).

infections, although with various sensitivity. In addition, they show no marked immune deficiencies against other pathogens.

In this study, we identified a novel mutation in *IFNGR1*, designated 774del4, in a patient with MSMD. Consistent with the results of a previous report,[23] the CD14-positive cells from this patient exhibited impaired production of TNFα in response to LPS and IFNγ, and the patient's CD3-positive cells did not produce IFNγ effectively in response to PHA and IL-12. It seems likely that the IFNγR1 deficiency not only impairs IFNγ signaling but also abolishes the Th1 cytokine cascade.

Based on these results, we examined the molecular basis for the dominant forms of IFNγR1 deficiency. We identified a 4 bp deletion, designated 774del4, in the *IFNGR1* gene of a patient with multiple osteomyelitic lesions due to *M. avium* infection. This was considered to be a sporadic case, as the same mutation was not detected in either of the patient's parents. The deletion causes a frameshift that introduces a premature stop codon (TGA) at nucleotides 827–829; the resulting truncated version of IFNγR1 lacks the expected intracellular domain. The 818del4 allele, which is the most frequent mutation observed in patients with the dominant form of IFNγR1 deficiency, also introduces a frameshift and premature stop codon at nucleotides 827–829.[4] Although the truncated protein encoded by 774del4 differs from that encoded by 818del4 in terms of the last 14 amino acids at the C-terminal end, patients with these mutations have similar clinical features.[5] Multifocal osteomyelitis caused by BCG or NTM in the absence of susceptibility to other infectious agents appears to be a characteristic feature of patients with either 818del4-related or 774del4-related IFNγR1 deficiency.

The protein encoded by the 774del4 allele of *IFNGR1* was overexpressed on the cell surface. To characterise this truncated version of IFNγR1, we cloned the WT allele and the 774del4 and 818del4 alleles of *IFNGR1* into a mammalian expression vector. Cycloheximide treatment enabled us to assess the stability of IFNγR1 expression by flow cytometry and Western blotting. In contrast to the WT protein, which disappeared rapidly even after cycloheximide treatment, the 774del4 and 818del4 mutant proteins were stably retained on the cell surface. These

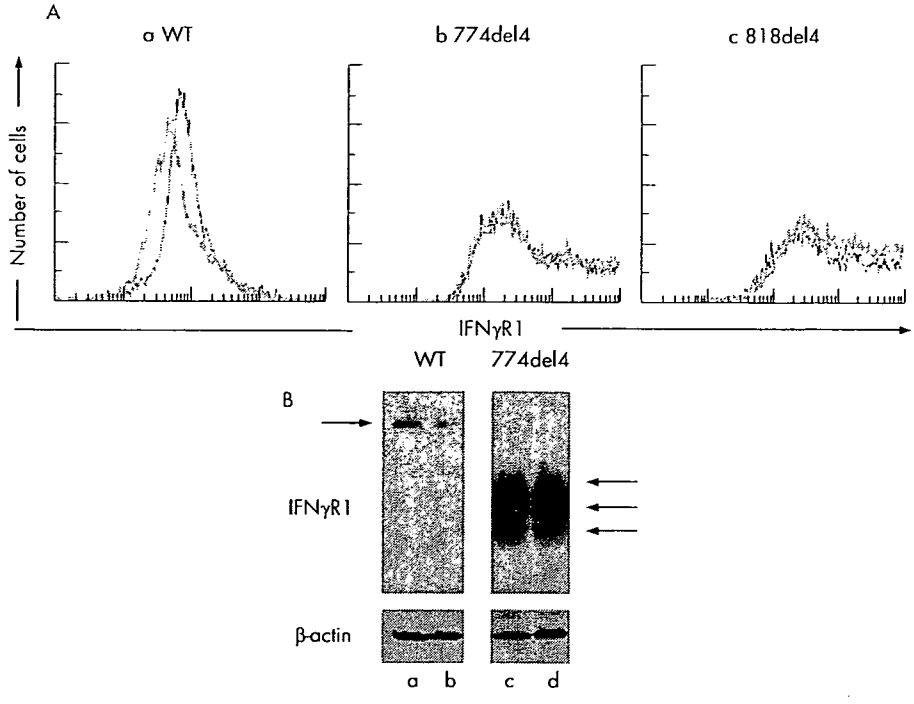


Figure 5 Stability of interferonγ receptor 1 (IFNγR1) in HEK 293 cells transfected with the wild-type (WT), 774del4 or 818del4 forms of *IFNGR1*. The WT, 774del4 and 818del4 *IFNGR1* cDNAs were introduced into the HEK 293 using the calcium phosphate method. At 24 hours after transfection, the cells were treated with 20 μg/ml cycloheximide for 3 hours. The expression levels of IFNγR1 were analysed by flow cytometry and Western blotting using monoclonal mouse antibodies against human IFNγR1. (A) IFNγR1 analysed by flow cytometry. (a) WT; (b) 774del4; (c) 818del4; solid line, cycloheximide-treated cells; dotted line, untreated cells. (B) The lysates of cells transfected with WT and 774del4 IFNγR1 were analysed by Western blotting. The cells were treated with CHX (a, c) or untreated (b, d).

observations suggest that both the 774del4 and 818del4 mutant forms of IFNγR1 are overexpressed on the cell surface, due to impaired receptor degradation. The intracellular domain of IFNγR1 contains two regions that are important for the receptor's function: the YDKPH sequence (residues 440–444), which is located near the C-terminus of IFNγR1 and is required for IFNγ-dependent cellular responses through STAT1 binding, and the membrane proximal region (residues 256–303), which is required for both receptor-mediated ligand internalisation and subsequent degradation, and for the induction of the biological response.[24 25] The importance of these regions is supported by radioligand binding analysis using both ^{125}I-labelled recombinant IFNγ and a series of IFNγR1 deletion mutants lacking the cytoplasmic region.[26] Without this region,

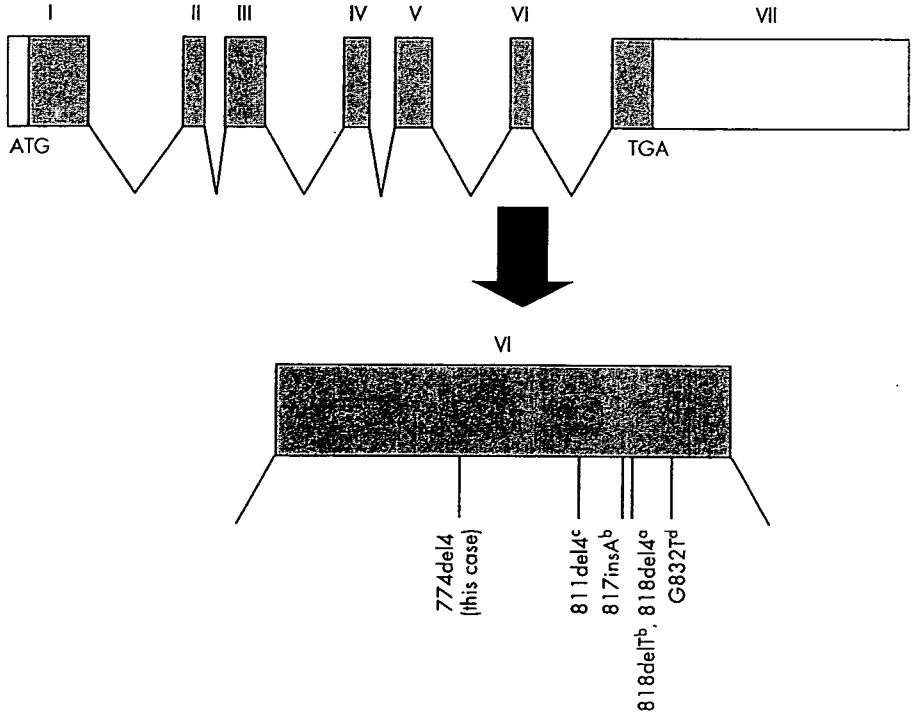


Figure 6 Summary of mutations in *IFNGR1* associated with dominant forms of IFNγR1 deficiency. The primary genomic structure of *IFNGR1*, including its seven exons, is shown, and all of the mutations previously shown to be associated with dominant forms of IFNγR1 deficiency are indicated. Specifically, the 774del4 mutation described in this report is depicted, along with the alleles described by Jouanguy *et al.*[a], Dorman *et al.*[b], Sasaki *et al.*[c], and Villella *et al.*[d] [4 12–15] All of the mutations are localised in exon 6. Unfilled boxes, non-coding regions; filled boxes, *IFNGR1* coding region.

both the internalation and degradation of IFNγ is impaired, and it accumulates at the cell surface.[18] Further analysis has revealed that a leucine–isoleucine motif (residues 270–271) near this region also plays an important role in receptor-mediated ligand internalisation/degradation and that the LPKS sequence (residues 266–269) is required for biological responses through JAK-1 binding.[24 27 28]

Jouanguy *et al.*[4] have suggested that the 818del4 mutation impairs the recycling of IFNγR1 because of the lack of the membrane proximal region, which results in cell-surface overexpression. This mutant receptor, which lacks both the LPKS and YDKPH motifs, is thought to impair IFNγ signalling via a dominant-negative effect; however, this assumption has not yet been examined at the molecular level. In this study, we examined the molecular mechanisms through which the 774del4 and 818del4 mutants exert dominant-negative effects on IFNγ signalling. In general, an mRNA with a premature stop codon tends to be rapidly degraded by the nonsense-mediated mRNA decay pathway.[29] However, both WT and mutant cDNAs were detected at similar ratios in the total RNA extracted from the peripheral blood of the patient, who carries both the WT and 774del4 mutant alleles (data not shown). Furthermore, we directly showed that the 774del4 and 818del4 mutant versions of IFNγR1 were stably overexpressed on the cell surface owing to alteration of receptor stability. Additionally, the CD14-positive cells from the patient showed a weaker response to IFNγ than did those from the healthy controls. Similarly, STAT1 phosphorylation in response to IFNγ was significantly impaired in cells derived from the patient. Together, these observations suggest that the 774del4 version of IFNγR1 is non-functional and has a dominant-negative effect on IFNγ signal transduction. To our knowledge, all of the mutations identified to date that cause dominant forms of IFNγR1 deficiency are localised in exon 6 of *IFNGR1* and lead to a premature stop in the intracellular domain (figure 6).

IFNγR1 deficiency is a primary immunodeficiency characterised by severe mycobacterial infections. Multifocal osteomyelitis without other organ involvement is an outstanding clinical feature of individuals with this deficiency.[5] Several patients with IFNγR1 deficiency who developed multiple osteomyelitis were initially misdiagnosed as having accompanying Langerhans cell histiocytosis and were treated with cytotoxic chemotherapy.[30] Of 50 previously reported patients with dominant forms of IFNγR1 deficiency, most were found to have the 818del4 mutation.[4 11] Sasaki *et al* reported four Japanese patients with dominant forms of *IFNGR1* deficiency from three unrelated families.[13] One of these patients was heterozygous for the 811del4 mutation, and the the other three were heterozygous for 818del4. The novel 774del4 mutation described in this study is the fifth allele shown to cause a dominant form of *IFNGR1* deficiency in Japan. Given that adjunctive therapeutic administration of IFNγ is beneficial for patients with dominant forms of IFNγR1 deficiency, more attention should be paid to this deficiency, especially when treating patients with recurrent mycobacterial infections.

Supplementary figure available on JMG website–http://jmg.bmj.com/supplemental

Authors' affiliations
Satoshi Okada, Nobutsune Ishikawa, Ken'ichiro Shirao, Hiroshi Kawaguchi, Masao Kobayashi, Department of Pediatrics, Hiroshima University Graduate School of Biomedical Science, Hiroshima, Japan
Miyuki Tsumura, Yoshinori Ohno, Shin'ichiro Yasunaga, Motoaki Ohtsubo, Yoshihiro Takihara, Department of Stem Cell Biology, Research Institute for Radiation Biology and Medicine, Hiroshima University

This work was supported by a grant-in-aid from the Japanese Ministry of Education, Culture, Sports, Science, and Technology and a grant-in-aid from the Japanese Ministry of Health, Labor, and Welfare. The 774del4 mutation of *IFNGR1* has been assigned GenBank accession number EF535103.

Competing interests: None declared.

REFERENCES

1 **Hamosh A,** Scott AF, Amberger JS, Bocchini CA, McKusick VA. Online Mendelian Inheritance in Man (OMIM), a knowledgebase of human genes and genetic disorders. *Nucleic Acids Res* 2005;**33**:D514–17.
2 **Newport MJ,** Huxley CM, Huston S, Hawrylowicz CM, Oostra BA, Williamson R, Levin M. A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection. *N Engl J Med* 1996;**335**:1941–9.
3 **Jouanguy E,** Altare F, Lamhamedi S, Revy P, Emile JF, Newport M, Levin M, Blanche S, Seboun E, Fischer A, Casanova JL. Interferon-gamma-receptor deficiency in an infant with fatal bacille Calmette-Guerin infection. *N Engl J Med* 1996;**335**:1956–61.
4 **Jouanguy E,** Lamhamedi-Cherradi S, Lammas D, Dorman SE, Fondaneche MC, Dupuis S, Doffinger R, Altare F, Girdlestone J, Emile JF, Ducoulombier H, Edgar D, Clarke J, Oxelius VA, Brai M, Novelli V, Heyne K, Fischer A, Holland SM, Kumararatne DS, Schreiber RD, Casanova JL. A human *IFNGR1* small deletion hotspot associated with dominant susceptibility to mycobacterial infection. *Nat Genet* 1999;**21**:370–8.
5 **Dorman SE,** Holland SM. Mutation in the signal-transducing chain of the interferon-gamma receptor and susceptibility to mycobacterial infection. *J Clin Invest* 1998;**101**:2364–9.
6 **Altare F,** Lammas D, Revy P, Jouanguy E, Doffinger R, Lamhamedi S, Drysdale P, Scheel-Toellner D, Girdlestone J, Darbyshire P, Wadhwa M, Dockrell H, Salmon M, Fischer A, Durandy A, Casanova JL, Kumararatne DS. Inherited interleukin 12 deficiency in a child with bacille Calmette-Guerin and Salmonella enteritidis disseminated infection. *J Clin Invest* 1998;**102**:2035–40.
7 **Altare F,** Durandy A, Lammas D, Emile JF, Lamhamedi S, Le Deist F, Drysdale P, Jouanguy E, Doffinger R, Bernaudin F, Jeppsson O, Gollob JA, Meinl E, Segal AW, Fischer A, Kumararatne D, Casanova JL. Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. *Science* 1998;**280**:1432–5.
8 **de Jong R,** Altare F, Haagen IA, Elferink DG, Boer T, van Breda Vriesman PJ, Kabel PJ, Draaisma JM, van Dissel JT, Kroon FP, Casanova JL, Ottenhoff TH. Severe mycobacterial and Salmonella infections in interleukin-12 receptor-deficient patients. *Science* 1998;**280**:1435–8.
9 **Dupuis S,** Dargemont C, Fieschi C, Thomassin N, Rosenzweig S, Harris J, Holland SM, Schreiber RD, Casanova JL. Impairment of mycobacterial but not viral immunity by a germline human STAT1 mutation. *Science* 2001;**293**:300–3.
10 **Filipe-Santos O,** Bustamante J, Haverkamp MH, Vinolo E, Ku CL, Puel A, Frucht DM, Christel K, von Bernuth H, Jouanguy E, Feinberg J, Durandy A, Senechal B, Chapgier A, Vogt G, de Beaucoudrey L, Fieschi C, Picard C, Garfa M, Chemli J, Bejaoui M, Tsolia MN, Kutukculer N, Plebani A, Notarangelo L, Bodemer C, Geissmann F, Israel A, Veron M, Knackstedt M, Barbouche R, Abel L, Magdorf K, Gendrel D, Agou F, Holland SM, Casanova JL. X-linked susceptibility to mycobacteria is caused by mutations in NEMO impairing CD40-dependent IL-12 production. *J Exp Med* 2006;**203**:1745–59.
11 **Filipe-Santos O,** Bustamante J, Chapgier A, Vogt G, de Beaucoudrey L, Feinberg J, Jouanguy E, Boisson-Dupuis S, Fieschi C, Picard C, Casanova JL. Inborn errors of IL-12/23- and IFN-gamma-mediated immunity: molecular, cellular, and clinical features. *Semin Immunol* 2006;**18**:347–61.
12 **Dorman SE,** Picard C, Lammas D, Heyne K, van Dissel JT, Baretto R, Rosenzweig SD, Newport M, Levin M, Roesler J, Kumararatne D, Casanova JL, Holland SM. Clinical features of dominant and recessive interferon gamma receptor 1 deficiencies. *Lancet* 2004;**364**:2113–21.
13 **Sasaki Y,** Nomura A, Kusuhara K, Takada H, Ahmed S, Obinata K, Hamada K, Okimoto Y, Hara T. Genetic basis of patients with bacille Calmette-Guérin osteomyelitis in Japan: identification of dominant partial interferon-gamma receptor 1 deficiency as a predominant type. *J Infect Dis* 2002;**185**:706–9.
14 **Villella A,** Picard C, Jouanguy E, Dupuis S, Popko S, Abughali N, Meyerson H, Casanova JL, Hostoffer RW. Recurrent *Mycobacterium avium* osteomyelitis associated with a novel dominant interferon gamma receptor mutation. *Pediatrics* 2001;**107**:e47.
15 **Dorman SE,** Uzel G, Roesler J, Bradley JS, Bastian J, Billman G, King S, Filie A, Schermerhorn J, Holland SM. Viral infections in interferon-gamma receptor deficiency. *J Pediatr* 1999;**135**:640–3.
16 **Luo P,** Tresini M, Cristofalo V, Chen X, Saulewicz A, Gray MD, Banker DE, Klingelhutz AL, Ohtsubo M, Takihara Y, Norwood TH. Immortalization in a normal foreskin fibroblast culture following transduction of cyclin A2 or cdk1 genes in retroviral vectors. *Exp Cell Res* 2004;**294**:406–19.
17 **Arend SM,** Janssen R, Gosen JJ, Waanders H, de Boer T, Ottenhoff TH, van Dissel JT. Multifocal osteomyelitis caused by nontuberculous mycobacteria in patients with a genetic defect of the interferon-gamma receptor. *Neth J Med* 2001;**59**:140–51.

18 **Waibel KH**, Regis DP, Uzel G, Rosenzweig SD, Holland SM. Fever and leg pain in a 42-month-old. *Ann Allergy Asthma Immunol* 2002;89:239–43.
19 **Raszka WV**, Trinh TT, Zawadsky PM. Multifocal M. intracellulare osteomyelitis in an immunocompetent child. *Clin Pediatr* 1994;33:611–16.
20 **Reiner SL**, Locksley RM. The regulation of immunity to Leishmania major. *Annu Rev Immunol* 1995;13:151–77.
21 **Magram J**, Connaughton SE, Warrier RR, Carvajal DM, Wu CY, Ferrante J, Stewart C, Sarmiento U, Faherty DA, Gately MK. IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses. *Immunity* 1996;4:471–81.
22 **Bustamante J**, Picard C, Fieschi C, Filipe-Santos O, Feinberg J, Perronne C, Chapgier A, de Beaucoudrey L, Vogt G, Sanlaville D, Lemainque A, Emile JF, Abel L, Casanova JL. A novel X-linked recessive form of Mendelian susceptibility to mycobaterial disease. *J Med Genet* 2007;44:e65.
23 **Feinberg J**, Fieschi C, Doffinger R, Feinberg M, Leclerc T, Boisson-Dupuis S, Picard C, Bustamante J, Chapgier A, Filipe-Santos O, Ku CL, de Beaucoudrey L, Reichenbach J, Antoni G, Balde R, Alcais A, Casanova JL. Bacillus Calmette Guerin triggers the IL-12/IFN-gamma axis by an IRAK-4- and NEMO-dependent, non-cognate interaction between monocytes, NK, and T lymphocytes. *Eur J Immunol* 2004;34:3276–84.
24 **Bach EA**, Aguet M, Schreiber RD. The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Annu Rev Immunol* 1997;15:563–91.
25 **Farrar MA**, Campbell JD, Schreiber RD. Identification of a functionally important sequence in the C terminus of the interferon-gamma receptor. *Proc Natl Acad Sci* 1992;89:11706–10.
26 **Farrar MA**, Fernandez-Luna J, Schreiber RD. Identification of two regions within the cytoplasmic domain of the human interferon-gamma receptor required for function. *J Biol Chem* 1991;266:19626–35.
27 **Kaplan DH**, Greenlund AC, Tanner JW, Shaw AS. 1996. Identification of an interferon-gamma receptor alpha chain sequence required for JAK-1 binding. *J Biol Chem* 1996;271:9–12.
28 **Farrar MA**, Schreiber RD. The molecular cell biology of interferon-gamma and its receptor. *Annu Rev Immunol* 1993;11:571–611.
29 **Maquat LE**. Nonsense-mediated mRNA decay: splicing, translation and mRNP dynamics. *Nat Rev Mol Cell Biol* 2004;5:89–99.
30 **Edgar JD**, Smyth AE, Pritchard J, Lammas D, Jouanguy E, Hague R, Novelli V, Dempsey S, Sweeney L, Taggart AJ, O'hara D, Casanova JL, Kumararatne DS. Interferon-gamma receptor deficiency mimicking Langerhans' cell histiocytosis. *J Pediatr* 2001;139:600–3.

Wiskott-Aldrich Syndrome is an Important Differential Diagnosis in Male Infants With Juvenile Myelomonocytic Leukemialike Features

Nobuhiro Watanabe, PhD, Ayami Yoshimi, PhD,† Yoshiro Kamachi, PhD,‡ Takashi Kawabe, MD,§ Hideki Muramatsu, MD,‡ Kimikazu Matsumoto, PhD,* Atsushi Manabe, PhD,‖ Seiji Kojima, PhD,‡ and Koji Kato, PhD**

Summary: A newborn presented with thrombocytopenia at birth and subsequently developed leukocytosis, monocytosis, and mild hepatomegaly. The bone marrow was normocellular with dysplasia and spontaneous granulocyte-monocyte colony formation was demonstrated. These findings fulfilled the diagnostic criteria of juvenile myelomonocytic leukemia. Then he developed atopic dermatitislike eczema, which led to the consideration of Wiskott-Aldrich syndrome (WAS). Lack of intracellular WASP expression and *WASP* gene mutation confirmed the diagnosis of WAS. After stem cell transplantation, he is alive in good condition with normal WASP expression. WAS should be considered as a differential diagnosis in male infants with juvenile myelomonocytic leukemialike features.

Key Words: Wiskott-Aldrich syndrome, juvenile myelomonocytic leukemia, male infants, differential diagnosis

(*J Pediatr Hematol Oncol* 2007;29:836–838)

Wiskott-Aldrich syndrome (WAS) is an X-linked disorder, characterized by thrombocytopenia with microplatelets, eczema, and recurrent infections, and affected patients have a predisposition to autoimmune disease and malignancy.[1] The responsible gene, *WASP*, encodes a 502 amino acid protein: WASP, that is expressed selectively in hematopoietic cells and is involved in cell signaling and cytoskeleton reorganization.[2] The identification of molecular defects led to broadening of the clinical spectrum of disorders associated with the *WASP* gene from classic severe WAS to a milder phenotype, X-linked thrombocytopenia. WASP expression is absent or reduced in these disorders. Interestingly, activating mutations of *WASP* were recently reported which cause X-linked neutropenia or myelodysplasia.[3,4]

Juvenile myelomonocytic leukemia (JMML) is a rare hematologic malignancy that accounts for approximately 1% of pediatric leukemias, having characteristics of both myelodysplastic syndrome and myeloproliferative disorders.[5,6] Clinical manifestations include hepatosplenomegaly, skin rash, lymphadenopathy, failure to thrive, leukoerythroblastosis, monocytosis ($>1\times10^9$/L), thrombocytopenia, and elevated fetal hemoglobin (HbF) levels. The diagnostic criteria proposed by the International JMML Working Group have been widely used.[7] Recent molecular studies have elucidated that the activated granulocyte macrophage colony-stimulating factor (GM-CSF)/Ras signaling pathway plays a central role in the pathogenesis of JMML. Somatic mutations in *PTPN11* and *RAS* are found in 34% and 25% of JMML patients, respectively.[8,9] Approximately, 25% of JMML cases are associated with neurofibromatosis type 1.[10] In summary, molecular abnormality of this pathway can be detected in up to 70% to 80% of JMML patients.

Here we report a male infant who fulfilled the diagnostic criteria of JMML and later demonstrated atopic dermatitislike eczema, which led to the diagnosis of WAS. The diagnosis was confirmed by flow cytometric analysis of intracellular WASP expression (FCM-WASP) and mutational analysis of the *WASP* gene. Although patients with WAS have a predisposition to hematologic malignancy, there are no previous reports on JMML-like features in patients with WAS. We suggest that WAS should be considered as one of the differential diagnoses in male infants presenting with JMML-like features, if no molecular markers of JMML can be demonstrated.

CASE REPORT

The patient is a male twin of a Japanese father and a Chinese mother, and the other twin suffered intrauterine death. Thrombocytopenia (platelet count: 42×10^9/L) was first

Received for publication April 23, 2007; accepted July 23, 2007.

From the *Division of Hematology and Oncology, Children's Medical Center, Japanese Red Cross Nagoya First Hospital; †Department of HSCT Data Management, Nagoya University, School of Medicine; ‡Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya; §Department of Pediatrics, Kasugai Municipal Hospital, Kasugai; and ‖Department of Pediatrics, St Luke's International Hospital, Tokyo, Japan.

Supported by a grant from the Ministry of Health, Labour, and Welfare of Japan, Tokyo.

Reprints: Nobuhiro Watanabe, PhD, Department of Pediatrics, Japanese Red Cross Nagoya First Hospital, 3-35 Michishita-cho, Nakamura-ku, Nagoya, Aichi 453-8511 Japan (e-mail: shudai@nagoyta-1st.jrc.or.jp).