suppressed following immunization in the OPN-siRNA-treated group at days 7 and 14 (Fig. 1).

3.2. OPN-siRNA reduced EAU scores

To investigate the potential of OPN-siRNA to prevent EAU, B6 mice were immunized with $hIRBP_{1-20}$ and treated twice with either OPN-siRNA or control-siRNA 24 h before and simultaneously with immunization. From day 7 after immunization, clinical assessment was performed every 3 or 4 days. As compared to the control group, the EAU clinical score was low in the OPN-siRNA-treated group during the entire period of observation (Fig. 2). In the OPN-siRNA-treated group, EAU reached a peak at day 28 after immunization. In contrast, control group mice peaked at day 21 (Fig. 2). The maximum clinical scores were significantly lower in the OPN-siRNA-treated group (average scores: 0.89 ± 0.68) than those in the control-siRNA-treated group (2.44 ± 0.78).

To further examine the effect of OPN-siRNA on EAU, histopathological examinations were performed. Eyes were removed from either OPN-siRNA- or control-siRNA-treated EAU mice 21 days after $hIRBP_{1-20}$ immunization. Representative histopathology of the eyes from mice treated with control-siRNA or OPN-siRNA is shown in Fig. 3A and B, respectively. In control mice, inflammatory cells were found in the retina, vitreous and choroid along with retinal folds and granulomatous lesions (Fig. 3A). Retinas collected from some mice treated with OPN-siRNA showed almost normal histology (Fig. 3B). The histological scores of retinal sections were significantly lower in OPN-siRNA-treated mice (average scores: 0.88 ± 0.69) than in control mice (1.5 ± 0.73; Fig. 3C).

3.3. OPN-siRNA treatment only partly reversed the disease

We next examined whether OPN-siRNA treatment could reverse an ongoing disease process. EAU was induced as usual by $hIRBP_{1-20}$

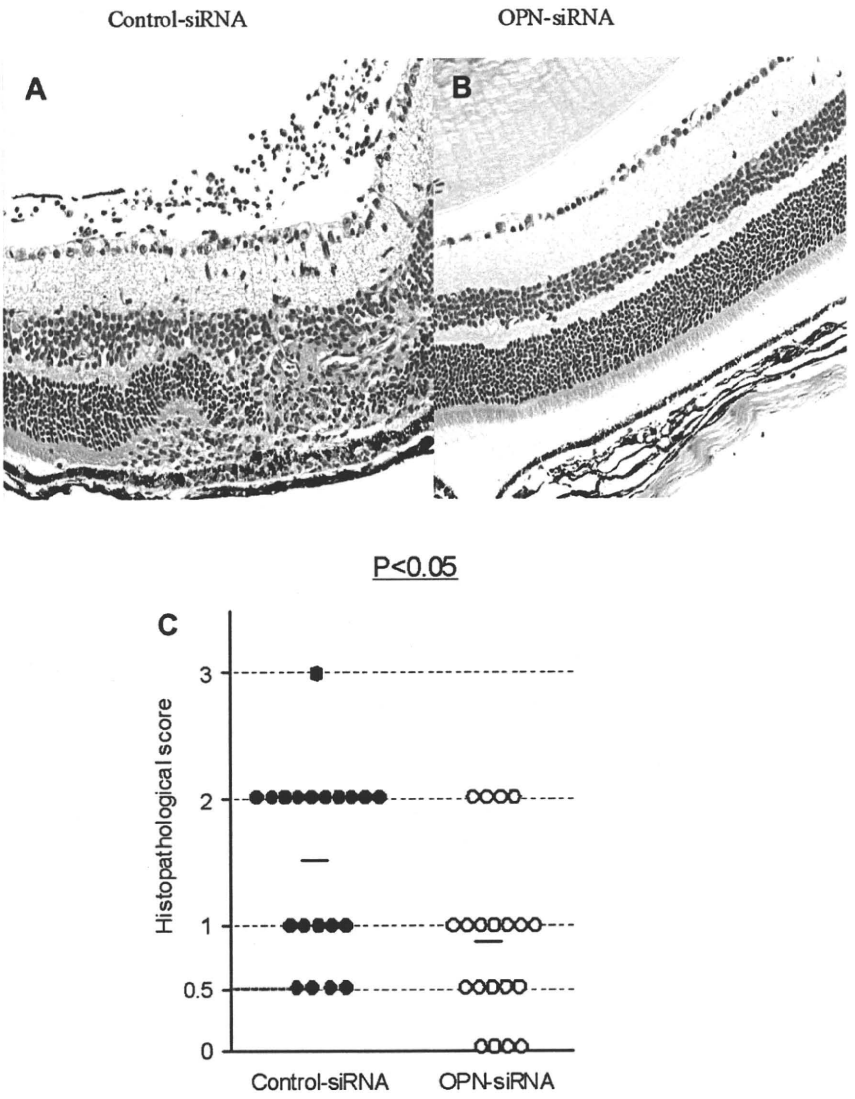

Fig. 3. Histopathology and histopathological score of EAU mice treated with OPN-siRNA. EAU was induced in B6 mice. Histopathology of mice treated with either control-siRNA (A) or OPN-siRNA (B). Note that inflammatory cells are present in the retina, vitreous, and choroid with retinal folds and granulomatous lesions in mice treated with control-siRNA (A) and the almost normal architecture of the retina in OPN-siRNA-treated mice (B). C. Mice were treated with OPN-siRNA (○) or control-siRNA (●). On day 21, the eyes were enucleated and scored by examining the histopathological sections of these eyes as shown in A and B. The results are presented as the histopathological score of each eye, and the mean EAU score of each group is indicated by a bar. Significance was determined by Mann–Whitney *U*-test ($P < 0.05$).

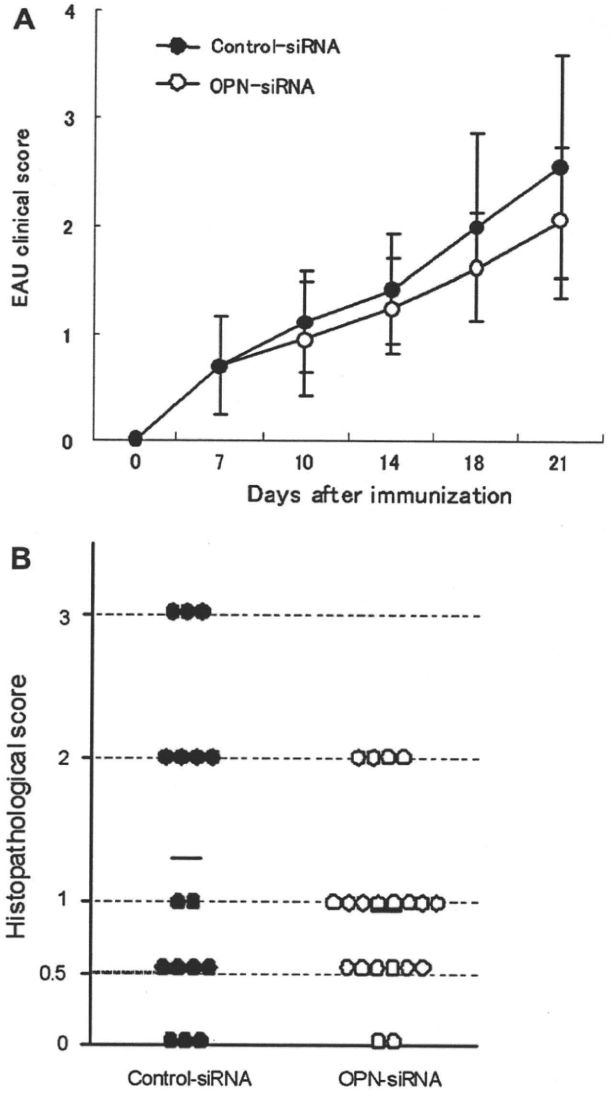

Fig. 4. Clinical and histopathological score of EAU in mice treated with OPN-siRNA with reversal regimen. A. EAU was induced by $hIRBP_{1-20}$ immunization at day 0. These mice were treated with two injections of either OPN-siRNA (○) or control-siRNA (●) on day 7 and day 8 after the immunization. Funduscopic examination was carried out every 3 or 4 days from day 7 after immunization. The results are presented as mean clinical score for all eyes of each group of mice (10 mice per group) ± standard deviation. Representative data of two separate experiments with similar results are presented. B. Histopathological score of EAU in mice treated with OPN-siRNA with reversal regimen. On day 21, the eyes from EAU mice were enucleated and scored of each eye. The mean EAU score of each group is indicated by a transverse bar.

immunization at day 0, and the mice were treated with two injections of either OPN- or control-siRNA at day 7 and day 8 when ocular symptoms first appeared overt after the immunization (reversal regimen). The clinical severity of EAU appeared to be slightly lower around day 21 but was not significantly reduced during the course of observation with the reversal regimen (Fig. 4A). The histopathological scores of retinal sections were not significantly lower in OPN-siRNA-treated mice (average scores: 0.95 ± 0.63) than in control mice (1.31 ± 1.11; Fig. 4B).

These results suggest that OPN-siRNA treatment more efficiently targets the priming rather than effector function of pathogenic T cells.

3.4. OPN-siRNA showed a slight influence on priming of hIRBP-specific T cells, but significantly inhibited Th1 and Th17 cytokine responses

To examine the mechanism underlying the suppressive effect of siRNA, we analyzed proliferative responses of lymphocytes from regional lymph nodes of hIRBP-immunized mice treated with OPN-siRNA or control-siRNA upon stimulation with hIRBP *in vitro*. As shown in Fig. 5A, lymphocytes from both groups mounted a considerable response. No significant differences were observed in the cell proliferation between OPN-siRNA-treated and control-siRNA-treated EAU mice, although the response in the OPN-siRNA group was slightly lower than that in the control group.

Next, we examined cytokine levels in the cultures of hIRBP peptide and lymphocytes collected from hIRBP-immunized mice treated with either OPN-siRNA or control-siRNA. We quantified IFN-γ and TNF-α concentrations in the culture supernatants. The levels of both IFN-γ and TNF-α were significantly reduced in the supernatants from cells of siRNA-treated mice compared to those of control-siRNA-treated mice at any concentrations of hIRBP analyzed (Fig. 5B). Furthermore, the production levels of IL-2, GM-CSF, and IL-17 were also significantly reduced in the supernatants from cells of OPN-siRNA-treated mice compared to those of control-siRNA-treated mice, whereas there no difference in the production of IL-1α, -4, 5, 6, and 10 between the two groups (Fig. 5C, and data not shown).

4. Discussion

In our previous study (Kitamura et al., 2007), we demonstrated that the plasma OPN levels were significantly elevated in EAU B6 mice by day 3 after immunization and peaked at day 14, which was concordant with the clinical course. Notably, OPN knockout (KO) mice displayed a considerably milder EAU and delayed disease onset compared with those of $OPN^{+/+}$ littermates (Hikita et al., 2006; Kitamura et al., 2007). In addition, EAU induced in B6 mice was ameliorated by administration of M5, an anti-OPN antibody. These findings demonstrated that OPN played a role in EAU development and might be an appropriate target for controlling ocular inflammation.

In the present study focusing on the blockade of OPN production, we used siRNA targeting the OPN coding sequence (OPN-siRNA). The OPN-siRNA was introduced into the animal with a hydrostatic pressure-mediated technique, hydrodynamic delivery (Liu et al., 1999). OPN is thought to function not only in soluble form (OPN-s) as a cytokine but also in intracellular form, OPN-i (Shinohara et al., 2006; Cantor and Shinohara, 2009). Although anti-OPN Ab can only be accessible to and block OPN-s, OPN-siRNA may block both forms by reducing the expression in both compartments.

First, we quantified the plasma level to evaluate the duration for the inhibition of OPN following *in vivo* siRNA treatment with the prevention regimen (day 1 and 0 of immunization) in EAU mice. OPN-siRNA treatment inhibited the increase of the plasma OPN level during the entire period of EAU to the basal level (Fig. 1). It was reported that OPN protein expression was significantly suppressed 5 days after *in vitro* siRNA treatment (Saito et al., 2007). In our study, OPN level remained significantly reduced at day 7 and day 14 in the OPN-siRNA-treated group (Fig. 1). This result suggested that OPN-siRNA treatment could have a longer period of efficacy than anticipated and thus may be applicable to chronic inflammatory diseases. When RNAi for OPN was induced with a prevention regimen, significant prevention of EAU was indeed manifested as had been shown with anti-OPN Ab (M5) treatment (Figs. 2 and 3). As to the clinical score, OPN-siRNA appeared to be more efficient than M5 (Kitamura et al., 2007).

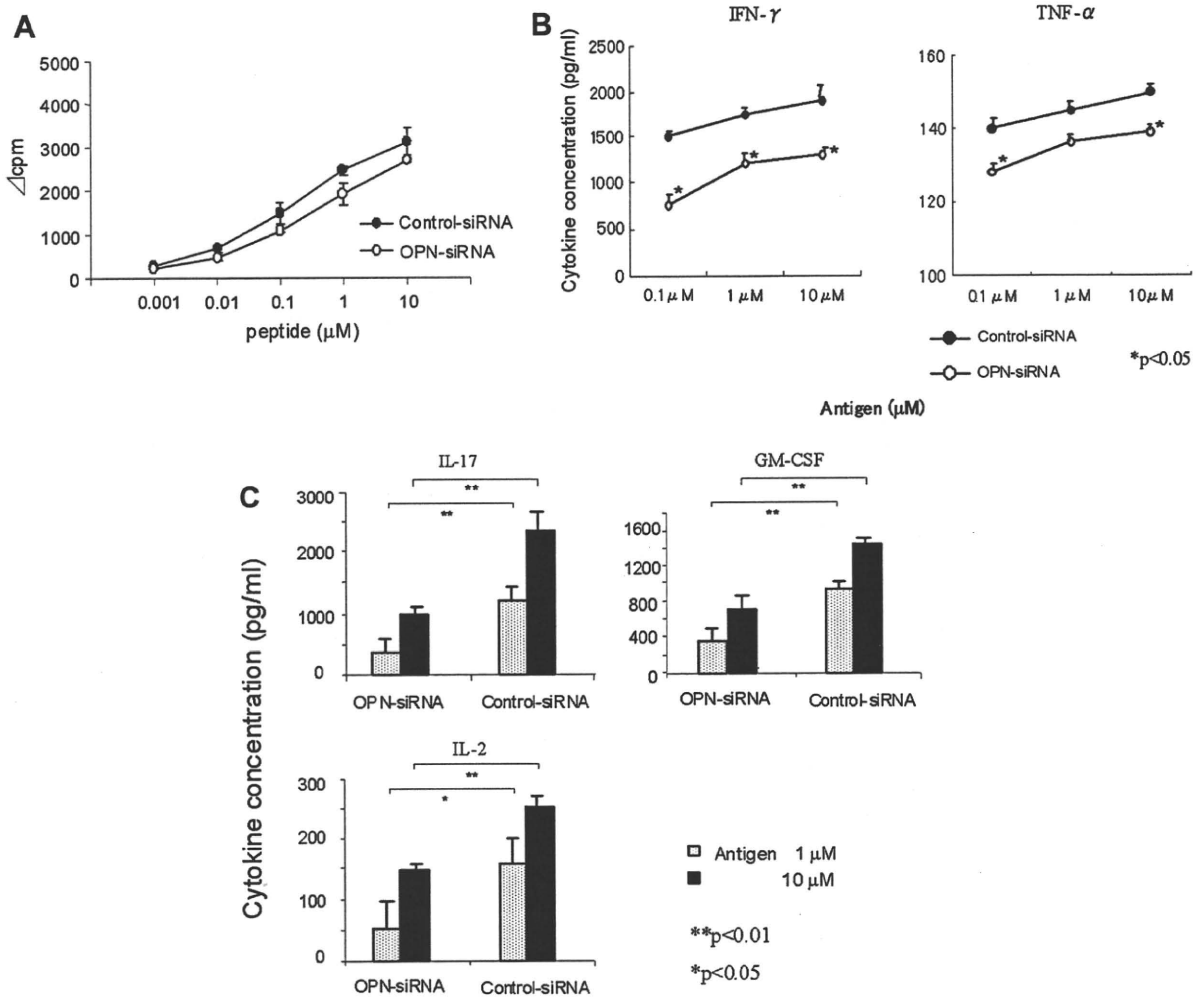

Fig. 5. Cell proliferative response and cytokine production of lymphocytes from regional lymph nodes of hIRBP-immunized and OPN-siRNA-treated mice. A. [^{3}H]-thymidine incorporation by primed lymphocytes. Lymphocytes were obtained from B6 mice immunized with hIRBP and treated with OPN-siRNA (●) or control-siRNA (○). Lymphocytes were incubated with indicated dose of hIRBP peptide and with [^{3}H]-thymidine for the last 16 h B. IFN-γ and TNF-α produced in the culture supernatant. C, IL-17, IL-2 and GM-CSF produced in the culture supernatant. The results are presented as mean ± standard deviation. Statistical significance is determined using two-tailed Student's *t*-test (**, $P < 0.01$, *, $P < 0.05$). Data are representative of two separate experiments with the similar result.

We also examined whether OPN-siRNA treatment could reverse ongoing EAU. Mice were immunized by $IRBP_{1-20}$ peptide at day 0 and treated with two injections of OPN-siRNA at day 7 and day 8 after EAU induction. At the 7-day time point, uveitogenic effector cells had already been primed and could induce EAU (Agarwal et al., 2000). As anticipated with this report, the reversal regimen was not effective for amelioration of the ongoing disease (Fig. 4A and B). These results suggested that the effects of OPN-siRNA were induced by the blockade of upsurge of OPN following immunization and thus preventing generation of primed T cells more than by inhibiting the effector function of induced T cells. However, the ineffectiveness of OPN blockade with siRNA after disease onset may not ruin its application for ongoing diseases. This is because the consecutive priming and generation of autoreactive T cells may take place even in chronic diseases. Moreover, co-administration of anti-OPN Ab may also compensate the effect of siRNA administration after onset, which should be pursued in further investigation.

We then examined the antigen-specific proliferative responses of lymphocytes upon *ex vivo* hIRBP peptide restimulation in the preventive regimen. Proliferation of hIRBP peptide-primed cells was slightly reduced by administration of OPN-siRNA *in vivo*. This finding is partially compatible with the result with the anti-OPN antibody (Kitamura et al., 2007). Notably, the production of IFN-γ and TNF-α was significantly reduced in the culture supernatants of the OPN-siRNA-treated group compared to that of the control group. The suppressed production of IFN-γ and TNF-α appeared to be interpreted by the blockade of Th1 cells, which led to the amelioration of EAU, a Th1-mediated autoimmune model (Caspi et al., 1988; Caspi, 2002; Schiffelers et al., 2005). IFN-γ induces macrophage activation and nitric oxide production, which leads to destruction of retina in EAU (Hoey et al., 1997). TNF-α provokes inflammatory responses (Green and Flavell, 1999) and TNF p55 receptor deficient mice are resistant to EAU (Calder et al., 2005).

OPN has been recognized as a key player in the Th1-responses for several reasons. First, the expression of OPN is mediated by T-bet, which is indispensable for the polarization of Th1 immune response (Shinohara et al., 2005). The secreted OPN further affects the expression of IL-12 (enhancement) and IL-10 (inhibition) that favors Th1-deviation (Ashkar et al., 2000). Second, the specific form of OPN, intracellular OPN, could induce IFN-α secretion from

plasmacytoid dendritic cells (pDC) in the presence of CpG in TLR9-MyD88- and IRF7-dependent manner (Shinohara et al., 2006). IFN-α also favors Th1-deviation. Third, OPN, especially the NH_2-terminal fragment of OPN cleaved by thrombin, promotes adhesion and migration of leukocytes and neutrophils and directly binds to $\alpha_9\beta_1$, which interacts with vascular cell adhesion molecule-1 (VCAM-1) in extravasation of neutrophils at sites of acute inflammation (Taooka et al., 1999). Thus, the migration of inflammatory cells might be blocked by the reduction of OPN content in the tissue.

It has been reported that OPN enhances survival of activated T cells by inhibiting transcription factor Foxo3a, activating NF-κB, and altering pro-apoptotic proteins (Hur et al., 2007). Thus, OPN function seems to be superfluous and not only supports Th1-deviation but also plays complex roles in immunological responses. This finding may explain the various influences on the manifestation of autoimmune diseases observed in different disease models.

On the other hand, there were conflicting results that EAU development was aggravated in IFN-γ KO and IFN-γ receptor KO mice (Fukushima et al., 2005; Hikita et al., 2006), which implied that IFN-γ might inhibit generation of pathogenic Th17 cells in EAU.

Recently, a new insight with Th17 cells has emerged for the pathogenesis of EAU (Amadi-Obi et al., 2007). In the present study, OPN-siRNA treatment suppressed not only Th1 cytokines but also IL-17 production, which could also account for the amelioration of EAU. These results suggest that OPN represents a good therapeutic target to ameliorate uveoretinitis as shown in our previous (Kitamura et al., 2007) and present studies. From a clinical viewpoint, OPN blockade seems to be not only potent but also beneficial for the treatment of human uveoretinitis without serious side effect, an obstacle of anti-TNF antibody therapy (Ohno et al., 2004). Thus far, no reports have demonstrated that OPN deficiency deteriorates host defense in mice (Rittling et al., 1998; Sato et al., 2005).

To date, OPN blockade has been shown to ameliorate various disease models in mice (Chabas et al., 2001; Jansson et al., 2002; Yumoto et al., 2002; Yamamoto et al., 2003; Hikita et al., 2006; Kitamura et al., 2007). Concordantly, OPN was elevated in human counterparts, including pulmonary sarcoidosis (Maeda et al., 2001), rheumatoid arthritis (Ohshima et al., 2002), and multiple sclerosis (Comabella et al., 2005). We thus presume that OPN blockade is also effective in these diseases. It is important to develop a safe and feasible technique for siRNA delivery to render the RNAi treatment applicable to human patients, as the hydrodynamic method is rather intense. Several novel techniques are being developed for the efficient introduction and interference for siRNA especially for *in vivo*-use (Liu et al., 1999; Howard and Kjems, 2007). The mechanistic elucidation and technical excellence will drive the RNAi for the treatment of immunological diseases with equal or better chance of use than monoclonal antibodies targeted to various molecules involved in disease development.

Disclosure

D. Iwata, None; M. Kitamura, None; N. Kitaichi, None; Y. Saito, None; S. Kon, None; K. Namba, None; J. Morimoto, None; A. Ebihara, None; H. Kitamei, None; K. Yoshida, None; S. Ishida, None; S. Ohno, None; T. Uede, None; K. Onoé, None; K. Iwabuchi, None.

References

Agarwal, R.K., Kang, Y., Zambidis, E., Scott, D.W., Chan, C.C., Caspi, R.R., 2000. Retroviral gene therapy with an immunoglobulin-antigen fusion construct protects from experimental autoimmune uveitis. J. Clin. Invest. 106, 245–252.

Amadi-Obi, A., Yu, C.R., Liu, X., Mahdi, R.M., Clarke, G.L., Nussenblatt, R.B., Gery, I., Lee, Y.S., Egwuagu, C.E., 2007. TH17 cells contribute to uveitis and scleritis and are expanded by IL-2 and inhibited by IL-27/STAT1. Nat. Med. 13, 711–718.

Ashkar, S., Weber, G.F., Panoutsakopoulou, V., Sanchirico, M.E., Jansson, M., Zawaideh, S., Rittling, S.R., Denhardt, D.T., Glimcher, M.J., Cantor, H., 2000. Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. Science 287, 860–864.

Calder, C.J., Nicholson, L.B., Dick, A.D., 2005. A selective role for the TNF p55 receptor in autocrine signaling following IFN-γ stimulation in experimental autoimmune uveoretinitis. J. Immunol. 175, 6286–6293.

Cantor, H., Shinohara, M.L., 2009. Regulation of T-helper-cell lineage development by osteopontin: the inside story. Nat. Rev. Immunol. 9, 137–141.

Caspi, R.R., 2002. Th1 and Th2 responses in pathogenesis and regulation of experimental autoimmune uveoretinitis. Int. Rev. Immunol. 21, 197–208.

Caspi, R.R., 2003. Experimental autoimmune uveoretinitis in the rat and mouse. Curr. Protoc. Immunol. Chapter 15: Unit 15.6.

Caspi, R.R., Roberge, F.G., Chan, C.C., Wiggert, B., Chader, G.J., Rozenszajn, L.A., Lando, Z., Nussenblatt, R.B., 1988. A new model of autoimmune disease. Experimental autoimmune uveoretinitis induced in mice with two different retinal antigens. J. Immunol. 140, 1490–1495.

Caspi, R.R., Roberge, F.G., McAllister, C.G., el-Saied, M., Kuwabara, T., Gery, I., Hanna, E., Nussenblatt, R.B., 1986. T cell lines mediating experimental autoimmune uveoretinitis (EAU) in the rat. J. Immunol. 136, 928–933.

Chabas, D., Baranzini, S.E., Mitchell, D., Bernard, C.C., Rittling, S.R., Denhardt, D.T., Sobel, R.A., Lock, C., Karpuj, M., Pedotti, R., Heller, R., Oksenberg, J.R., Steinman, L., 2001. The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease. Science 294, 1731–1735.

Comabella, M., Pericot, I., Goertsches, R., Nos, C., Castillo, M., Blas Navarro, J., Rio, J., Montalban, X., 2005. Plasma osteopontin levels in multiple sclerosis. J. Neuroimmunol. 158, 231–239.

Cook, E.B., Stahl, J.L., Lowe, L., Chen, R., Morgan, E., Wilson, J., Varro, R., Chan, A., Graziano, F.M., Barney, N.P., 2001. Simultaneous measurement of six cytokines in a single sample of human tears using microparticle-based flow cytometry: allergics vs. non-allergics. J. Immunol. Methods 254, 109–118.

Denhardt, D.T., Noda, M., O'Regan, A.W., Pavlin, D., Berman, J.S., 2001. Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. J. Clin. Invest. 107, 1055–1061.

Diao, H., Kon, S., Iwabuchi, K., Kimura, C., Morimoto, J., Ito, D., Segawa, T., Maeda, M., Hamuro, J., Nakayama, T., Taniguchi, M., Yagita, H., Van Kaer, L., Onoe, K., Denhardt, D., Rittling, S., Uede, T., 2004. Osteopontin as a mediator of NKT cell function in T cell-mediated liver diseases. Immunity 21, 539–550.

Diao, H., Iwabuchi, K., Li, L., Onoé, L., Van Kaer, L., Kon, S., Saito, Y., Morimoto, J., Denhardt, D., Rittling, S., Uede, T., 2008. Osteopontin regulates development and function of invariant natural killer T cells. Proc. Natl. Acad. Sci. USA 105, 15884–15889.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T., 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494–498.

Fukushima, A., Yamaguchi, T., Ishida, W., Fukata, K., Udaka, K., Ueno, H., 2005. Mice lacking the IFN-gamma receptor or fyn develop severe experimental autoimmune uveoretinitis characterized by different immune responses. Immunogenetics 57, 337–343.

Green, E.A., Flavell, R.A., 1999. Tumor necrosis factor-α and the progression of diabetes in non-obese diabetic mice. Immunol. Rev. 169, 11–22.

Gregerson, D.S., Obritsch, W.F., Fling, S.P., Cameron, J.D., 1986. S-antigen-specific rat T cell lines recognize peptide fragments of S-antigen and mediate experimental autoimmune uveoretinitis and pinealitis. J. Immunol. 136, 2875–2882.

Hannon, G.J., 2002. RNA interference. Nature 418, 244–251.

Hikita, S.T., Vistica, B.P., Jones, H.R., Keswani, J.R., Watson, M.M., Ericson, V.R., Ayoub, G.S., Gery, I., Clegg, D.O., 2006. Osteopontin is proinflammatory in experimental autoimmune uveitis. Invest. Ophthalmol. Vis. Sci. 47, 4435–4443.

Hoey, S., Grabowski, P.S., Ralston, S.H., Forrester, J.V., Liversidge, J., 1997. Nitric oxide accelerates the onset and increases the severity of experimental autoimmune uveoretinitis through an IFN-γ-dependent mechanism. J. Immunol. 159, 5132–5142.

Howard, K.A., Kjems, J., 2007. Polycation-based nanoparticle delivery for improved RNA interference therapeutics. Expert Opin. Biol. Ther. 7, 1811–1822.

Hur, E.M., Youssef, S., Haws, M.E., Zhang, S.Y., Sobel, R.A., Steinman, L., 2007. Osteopontin-induced relapse and progression of autoimmune brain disease through enhanced survival of activated T cells. Nat. Immunol. 8, 74–83.

Jansson, M., Panoutsakopoulou, V., Baker, J., Klein, L., Cantor, H., 2002. Cutting edge: attenuated experimental autoimmune encephalomyelitis in eta-1/osteopontin-deficient mice. J. Immunol. 168, 2096–2099.

Jiang, H.R., Lumsden, L., Forrester, J.V., 1999. Macrophages and dendritic cells in IRBP-induced experimental autoimmune uveoretinitis in B10RIII mice. Invest. Ophthalmol. Vis. Sci. 40, 3177–3185.

Kezuka, T., Sakai, J., Yokoi, H., Takeuchi, M., Okada, A., Taguchi, O., Usui, M., Mizuguchi, J., 1996. Peptide-mediated suppression of experimental autoimmune uveoretinitis in mice: development of a peptide vaccine. Int. Immunol. 8, 1229–1235.

Khoury, M., Louis-Plence, P., Escriou, V., Noel, D., Largeau, C., Cantos, C., Scherman, D., Jorgensen, C., Apparailly, F., 2006. Efficient new cationic liposome formulation for systemic delivery of small interfering RNA silencing tumor necrosis factor alpha in experimental arthritis. Arthritis Rheum. 54, 1867–1877.

Kitamura, M., Iwabuchi, K., Kitaichi, N., Kon, S., Kitamei, H., Namba, K., Yoshida, K., Denhardt, D.T., Rittling, S.R., Ohno, S., Uede, T., Onoe, K., 2007. Osteopontin aggravates experimental autoimmune uveoretinitis in mice. J. Immunol. 178, 6567–6572.

Liu, F., Song, Y., Liu, D., 1999. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Ther. 6, 1258–1266.

Luger, D., Silver, P.B., Tang, J., Cua, D., Chen, Z., Iwakura, Y., Bowman, E.P., Sgambellone, N.M., Chan, C.C., Caspi, R.R., 2008. Either a Th17 or a Th1 effector

response can drive autoimmunity: conditions of disease induction affect dominant effector category. J. Exp. Med. 205, 799–810.
Maeda, K., Takahashi, K., Takahashi, F., Tamura, N., Maeda, M., Kon, S., Uede, T., Fukuchi, Y., 2001. Distinct roles of osteopontin fragments in the development of the pulmonary involvement in sarcoidosis. Lung 179, 279–291.
Mochizuki, M., Kuwabara, T., McAllister, C., Nussenblatt, R.B., Gery, I., 1985. Adoptive transfer of experimental autoimmune uveoretinitis in rats. Immunopathogenic mechanisms and histologic features. Invest. Ophthalmol. Vis. Sci. 26, 1–9.
Nakamura, H., Siddiqui, S.S., Shen, X., Malik, A.B., Pulido, J.S., Kumar, N.M., Yue, B.Y., 2004. RNA interference targeting transforming growth factor-beta type II receptor suppresses ocular inflammation and fibrosis. Mol. Vis. 10, 703–711.
Namba, K., Ogasawara, K., Kitaichi, N., Morohashi, T., Sasamoto, Y., Kotake, S., Matsuda, H., Iwabuchi, K., Iwabuchi, C., Ohno, S., Onoe, K., 2000. Amelioration of experimental autoimmune uveoretinitis by pretreatment with a pathogenic peptide in liposome and anti-CD40 ligand monoclonal antibody. J. Immunol. 165, 2962–2969.
Ohno, S., Nakamura, S., Hori, S., Shimakawa, S., Kawashima, H., Mochizuki, M., Sugita, S., Ueno, S., Yoshizaki, K., Inaba, G., 2004. Efficacy, safety, and pharmakokinetics of multiple administration of infliximab in Behçet's disease with refractory uveoretinitis. J. Rheumatol. 31, 1362–1368.
Ohshima, S., Yamaguchi, N., Nishioka, K., Mima, T., Ishii, T., Umeshita-Sasai, M., Kobayashi, H., Shimizu, M., Katada, Y., Wakitani, S., Murata, N., Nomura, S., Matsuno, H., Katayama, R., Kon, S., Inobe, M., Uede, T., Kawase, I., Saeki, Y., 2002. Enhanced local production of osteopontin in rheumatoid joints. J. Rheumatol. 29, 2061–2067.
O'Regan, A., Berman, J.S., 2000. Osteopontin: a key cytokine in cell-mediated and granulomatous inflammation. Int. J. Exp. Pathol. 81, 373–390.
Rittling, S.R., Matsumoto, H.N., McKee, M.D., Nanci, A., An, X.R., Novick, K.E., Kowalski, A.J., Noda, M., Denhardt, D.T., 1998. Mice lacking osteopontin show normal development and bone structure but display altered osteoclast formation in vitro. J. Bone Miner. Res. 13, 1101–1111.
Saito, Y., Kon, S., Fujiwara, Y., Nakayama, Y., Kurotaki, D., Fukuda, N., Kimura, C., Kanayama, M., Ito, K., Diao, H., Matsui, Y., Komatsu, Y., Ohtsuka, E., Uede, T., 2007. Osteopontin small interfering RNA protects mice from fulminant hepatitis. Hum. Gene Ther. 18, 1205–1214.
Saoudi, A., Kuhn, J., Huygen, K., de Kozak, Y., Velu, T., Goldman, M., Druet, P., Bellon, B., 1993. TH2 activated cells prevent experimental autoimmune uveoretinitis, a TH1-dependent autoimmune disease. Eur. J. Immunol. 23, 3096–3103.
Sato, I., Yamamoto, N., Yamazaki, H., Hashimoto, S., Hino, M., Sakai, F., Fujie, A., 2005. Prevention of the cryptic epitope SLAYGLR within osteopontin does not influence susceptibility to *Candida albicans* infection. Antimicrob. Agents Chemother. 49, 3053–3055.
Schiffelers, R.M., Xu, J., Storm, G., Woodle, M.C., Scaria, P.V., 2005. Effects of treatment with small interfering RNA on joint inflammation in mice with collagen-induced arthritis. Arthritis Rheum. 52, 1314–1318.
Shinohara, M.L., Jansson, M., Hwang, E.S., Werneck, M.B., Glimcher, L.H., Cantor, H., 2005. T-bet-dependent expression of osteopontin contributes to T cell polarization. Proc. Natl. Acad. Sci. USA 102, 17101–17106.
Shinohara, M.L., Lu, L., Bu, J., Werneck, M.B., Kobayashi, K.S., Glimcher, L.H., Cantor, H., 2006. Osteopontin expression is essential for interferon-α production by plasmacytoid dendritic cells. Nat. Immunol. 7, 498–506.
Silver, P.B., Chan, C.C., Wiggert, B., Caspi, R.R., 1999. The requirement for pertussis to induce EAU is strain-dependent: B10.RIII, but not B10.A mice, develop EAU and Th1 responses to IRBP without pertussis treatment. Invest. Ophthalmol. Vis. Sci. 40, 2898–2905.
Song, E., Lee, S.K., Wang, J., Ince, N., Ouyang, N., Min, J., Chen, J., Shankar, P., Lieberman, J., 2003. RNA interference targeting Fas protects mice from fulminant hepatitis. Nat. Med. 9, 347–351.
Taooka, Y., Chen, J., Yednock, T., Sheppard, D., 1999. The integrin alpha9beta1 mediates adhesion to activated endothelial cells and transendothelial neutrophil migration through interaction with vascular cell adhesion molecule-1. J. Cell Biol. 145, 413–420.
Thurau, S.R., Chan, C.C., Nussenblatt, R.B., Caspi, R.R., 1997. Oral tolerance in a murine model of relapsing experimental autoimmune uveoretinitis (EAU): induction of protective tolerance in primed animals. Clin. Exp. Immunol. 109, 370–376.
Xie, F.Y., Woodle, M.C., Lu, P.Y., 2006. Harnessing in vivo siRNA delivery for drug discovery and therapeutic development. Drug Discov. Today 11, 67–73.
Yamamoto, N., Sakai, F., Kon, S., Morimoto, J., Kimura, C., Yamazaki, H., Okazaki, I., Seki, N., Fujii, T., Uede, T., 2003. Essential role of the cryptic epitope SLAYGLR within osteopontin in a murine model of rheumatoid arthritis. J. Clin. Invest. 112, 181–188.
Yumoto, K., Ishijima, M., Rittling, S.R., Tsuji, K., Tsuchiya, Y., Kon, S., Nifuji, A., Uede, T., Denhardt, D.T., Noda, M., 2002. Osteopontin deficiency protects joints against destruction in anti-type II collagen antibody-induced arthritis in mice. Proc. Natl. Acad. Sci. USA 99, 4556–4561.

nature genetics

Genome-wide association studies identify *IL23R-IL12RB2* and *IL10* as Behçet's disease susceptibility loci

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Behçet's disease is a chronic systemic inflammatory disorder characterized by four major manifestations: recurrent ocular symptoms, oral and genital ulcers and skin lesions[1]. We conducted a genome-wide association study in a Japanese cohort including 612 individuals with Behçet's disease and 740 unaffected individuals (controls). We identified two suggestive associations on chromosomes 1p31.3 (*IL23R-IL12RB2*, rs12119179, $P = 2.7 \times 10^{-8}$) and 1q32.1 (*IL10*, rs1554286, $P = 8.0 \times 10^{-8}$). A meta-analysis of these two loci with results from additional Turkish and Korean cohorts showed genome-wide significant associations (rs1495965 in *IL23R-IL12RB2*, $P = 1.9 \times 10^{-11}$, odds ratio = 1.35; rs1800871 in *IL10*, $P = 1.0 \times 10^{-14}$, odds ratio = 1.45).

Behçet's disease exists worldwide but is more prevalent in countries along the ancient silk route spanning from Japan to the Middle East and the Mediterranean basin[2]. Although the etiology of the disease remains poorly characterized, it is currently thought, as for many autoimmune or autoinflammatory syndromes, that certain environmental factors are able to trigger symptomatology in individuals with particular genetic variants. For the most part, the nature of these genetic variants remains unknown, with the exception of the historically known association with the human leukocyte antigen (HLA) class I region[2], in which the *HLA-B*51* allele itself or a closely linked gene (for example, *MICA*) is associated with Behçet's disease. More recently, we performed a genome-wide association study (GWAS) for Behçet's disease using microsatellite markers and were able to show a split in the HLA contribution to disease, that is, the *HLA-A*26* allele is associated with Behçet's disease independently of *HLA-B*51* (ref. 3).

Although a previous study also performed a preliminary GWAS using the DNA pooling method with the Affymetrix 500K arrays in Turkish individuals and reported several non-*HLA* susceptibility loci and genes for Behçet's disease[4], their study was limited by mapping resolution and low detection power.

Here we revisit this same issue using a larger sample pool—often a challenge to find for rare disorders—to identify susceptibility genes for Behçet's disease beyond the *HLA* complex. We conducted a GWAS in Japanese populations using 500,568 SNPs from the Affymetrix GeneChip Human Mapping 500K Array Set. We genotyped 612 Japanese individuals with Behçet's disease (cases) and 740 unaffected controls. After sample and SNP quality control, we analyzed a total of 320,438 SNPs in 611 cases and 737 controls (Online Methods and **Supplementary Table 1**). Principal component analysis showed no evidence of population admixture in the GWAS cohort. We further calculated the genomic inflation factor (λ) in this cohort; which was 1.05.

The *HLA-B* region showed the most significant association with Behçet's disease (rs4959053, $P = 1.8 \times 10^{-26}$), and we observed genome-wide significant signals ($P < 5.0 \times 10^{-8}$) for 80 SNPs in the *HLA* complex; many of these SNPs neighbored *HLA-A* and *HLA-B* genes in the *HLA* class I region (**Fig. 1** and **Supplementary Table 2**). To further dissect Behçet's disease susceptibility in the *HLA* class I region, we investigated linkage disequilibrium (LD) patterns in the region and performed association analysis in the *HLA-A* subregion according to whether or not *HLA-B*51* was present. As previously reported[3], we confirmed an independent contribution of two *HLA* subregions, *HLA-B* and *HLA-A*, to the risk of Behçet's disease in this Japanese cohort, which includes the 300 cases and 300 controls used in our previous GWAS (A.M., data not shown).

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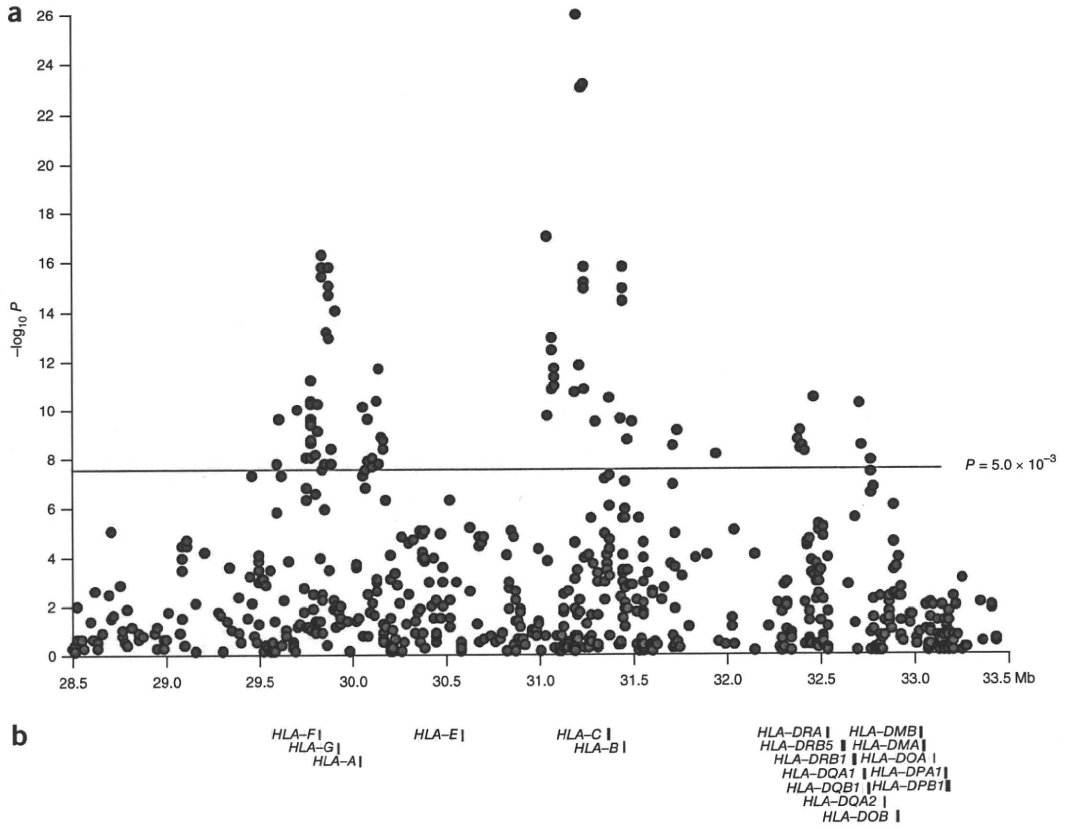

Figure 1 Overview of SNPs across the *HLA* region in the GWAS. (**a**) Association of SNPs located at 28.5–33.5 Mb on chromosome 6. Genotyped SNPs in the GWAS are indicated with a blue circle. (**b**) Location of *HLA* genes from the NCBI RNA reference sequences collection at 28.5–33.5 Mb on chromosome 6.

Outside the *HLA* complex, a total of 54 SNPs at 35 distinct loci showed association with Behçet's disease at $P < 0.0001$ in allele-based tests (**Supplementary Table 3**). Among these SNPs, three of the top four were located on chromosome 1p31.33 and the third was located on chromosome 1q32.1. Genome-wide significant association (using the threshold of $P < 5.0 \times 10^{-8}$) was detected at $P = 2.7 \times 10^{-8}$ for rs12119179, which is located in the 47-kb intergenic region between *IL23R* (encoding interleukin 23 receptor) and *IL12RB2* (encoding interleukin 12 receptor, beta 2). The second strongest region of association was rs1554286, located within the intron 3 of *IL10* (encoding interleukin 10) and also on chromosome 1q32.1 ($P = 8.0 \times 10^{-8}$).

To further validate our GWAS results, we exchanged data with colleagues performing a similar GWAS in a Turkish population[5]. This independent analysis also showed a strong dichotomized association of Behçet's disease with *HLA-B* and *HLA-A* loci in the *HLA* region. In addition, this Turkish GWAS identified the associations at the same two non-*HLA* regions, *IL10* and *IL23R-IL12RB2*, with $P < 0.0001$. This provides cross-validation of these two associations in an independent cohort from a distinct population.

We then fine mapped the association at the *IL23R-IL12RB2* and *IL10* loci using additional SNPs. For the *IL23R-IL12RB2* loci, we genotyped an additional set of 26 SNPs in the 230-kb region covering these genes. Of these SNPs, 23 were genotyped successfully, and 3 were monomorphic. We observed significant associations for several SNPs. The most significant was observed with rs1495965, located in the intergenic region between *IL23R* and *IL12RB2* ($P = 1.2 \times 10^{-8}$) (**Fig. 2a**, **Table 1** and **Supplementary Table 4**). SNPs with strong association in fine mapping or at the GWAS stage (rs11209032, rs11209033, rs12141431, rs12119179 and rs1495965) were in strong LD with each other, and this LD block was located in the intergenic region between *IL23R* and *IL12RB2*. In addition, outside this block, we found a weak association for several SNPs in *IL23R* and for one SNP in *IL12RB2*. To fine-map the *IL10* locus, we genotyped an additional set of 11 SNPs in the 8-kb region covering the gene. All SNPs were genotyped successfully, and we observed strong associations for four SNPs: rs1518111, rs3024490, rs1800872 and rs1800871. The most significant association ($P = 9.5 \times 10^{-9}$) was observed at rs1800872 and rs1800871, located in the promoter region of *IL10* (**Fig. 2b**, **Table 1** and **Supplementary Table 5**). These four SNPs and rs1554286 (found in the initial GWAS) were in strong LD with each other.

Finally, we evaluated the three SNPs showing the most significant *P* values (rs1495965 in *IL23R-IL12RB2* and rs1800872 and rs1800871 in *IL10*) for replication in 2,494 Turkish individuals (obtained from colleagues performing a parallel GWAS in a Turkish population and reported in a coordinated publication in this issue[5]) and 259 Korean individuals (**Table 1**). rs1495965 in *IL23R-IL12RB2* did show evidence of replication in the Turkish cohort ($P = 8.4 \times 10^{-5}$), but this SNP was not significantly associated with Behçet's disease in the Korean cohort ($P = 0.094$). The G allele of rs1495965 showed an increased risk of

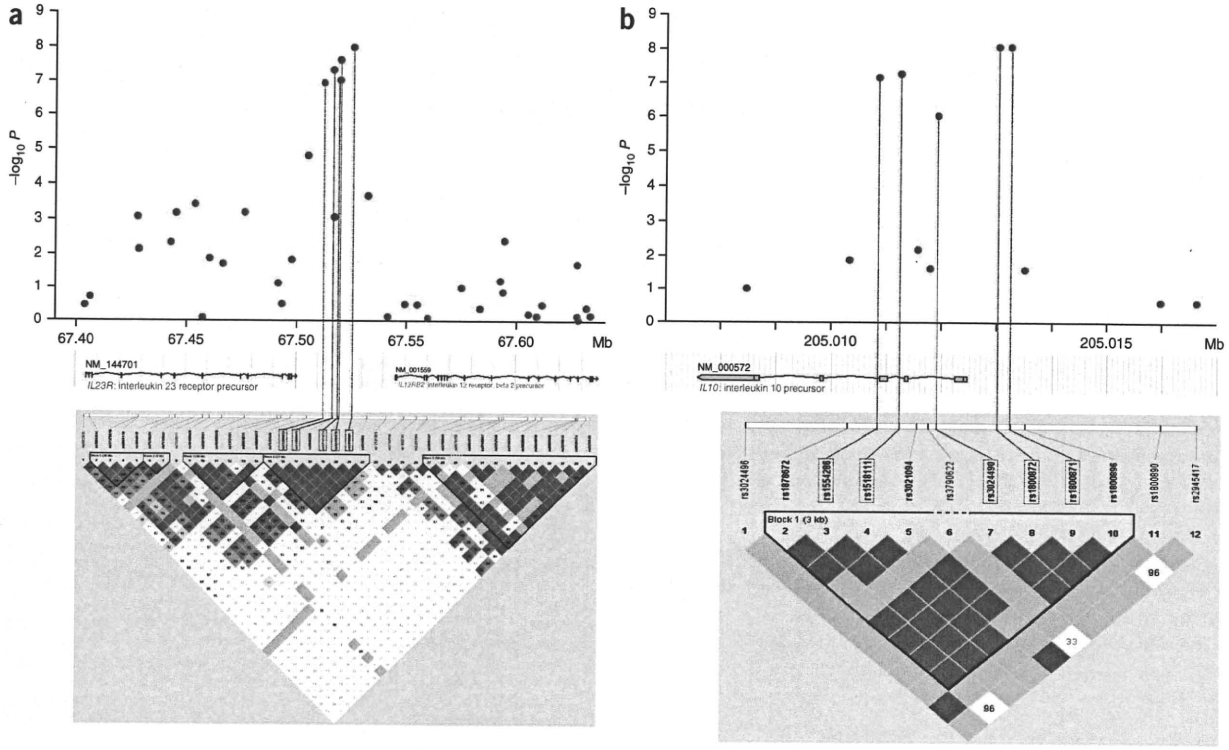

Figure 2 In-depth SNP analysis in target areas. (**a**) Association analysis of SNPs across *IL23R* and *IL12RB2*. (**b**) Association analysis of SNPs across *IL10*. The upper panels show distribution of association results of SNPs in the target gene regions. Genotyped SNPs in the GWAS are indicated with a blue circle, and genotyped SNPs found through fine mapping are indicated with a red circle. The lower panels show LD structure in the target gene regions; higher *D'* is indicated by a brighter red. The yellow and gray segments within exon boxes indicate the coding and untranslated regions, respectively.

Behçet's disease in the three populations ($P = 1.9 \times 10^{-11}$, odds ratio (OR) = 1.35, 95% CI 1.24–1.47). rs1800872 and rs1800871 in *IL10* replicated in both the Turkish and Korean cohorts, and the results from the meta-analysis showed the most significant association (rs1800872, $P = 2.1 \times 10^{-14}$, OR = 1.45, 95% CI 1.32–1.59; rs1800871, $P = 1.0 \times 10^{-14}$, OR = 1.45, 95% CI 1.32–1.60).

Recent genetic surveys including GWAS have identified *IL23R* or *IL12RB2* as susceptibility loci for a number of inflammatory and immune-linked diseases[6–11]. For example, the non-synonymous SNP rs11209026, located within exon 9 of *IL23R*, is primarily associated with inflammatory bowel disease, psoriasis, psoriatic arthritis and ankylosing spondylitis. Although rs11209026 was monomorphic in the Japanese populations, two SNPs in the Behçet's disease–associated LD block, rs11209032 and rs1495965, and other two Behçet's disease–associated SNPs with $P < 0.01$, rs1004819 and rs7517847, have been reported to be associated with inflammatory bowel disease. *IL23R*

Table 1 Allelic association results for SNPs rs1495965, rs1800872 and rs1800871

SNP	Chr. position[a]	Nearest gene	Risk allele	Population	*N*		Risk allele frequency		P^b	OR for risk allele (95% CI)
					Cases	Controls	Cases	Controls		
rs1495965	67,526,096	*IL23R-IL12RB2*	G	Japanese	611	737	0.563	0.451	1.2×10^{-8}	1.56 (1.34–1.83)
				Turkish	1,215	1,279	0.568	0.512	8.4×10^{-5}	1.25 (1.12–1.40)
				Korean	119	140	0.560	0.486	0.094	1.35 (0.95–1.91)
				Overall					1.9×10^{-11}	1.35 (1.24–1.47)
rs1800872	205,013,030	*IL10*	A	Japanese	611	737	0.765	0.664	9.5×10^{-9}	1.64 (1.39–1.95)
				Turkish	1,215	1,279	0.384	0.315	6.1×10^{-7}	1.35 (1.20–1.52)
				Korean	119	140	0.776	0.694	0.038	1.52 (1.02–2.28)
				Overall					2.1×10^{-14}	1.45 (1.32–1.59)
rs1800871	205,013,257	*IL10*	T	Japanese	611	737	0.765	0.664	9.5×10^{-9}	1.64 (1.39–1.95)
				Turkish	1,215	1,279	0.383	0.313	3.1×10^{-7}	1.36 (1.21–1.54)
				Korean	119	140	0.774	0.694	0.044	1.51 (1.01–2.25)
				Overall					1.0×10^{-14}	1.45 (1.32–1.60)

Chr., chromosome.
[a]NCBI build 36; all SNPs were located on chromosome 1. [b]*P* values for each population were calculated using an allele-based test; overall *P* values and ORs in three populations were calculated using the Mantel-Haenzel method.

encodes a subunit of the IL23 receptor. IL23 is a heterodimeric pro-inflammatory cytokine composed of a p19 subunit and a p40 subunit that is shared with IL12. IL23 has been shown to stimulate T helper 17 cell proliferation and increase the production of inflammatory cytokines such as IL1, IL6, IL17 and TNFα (ref. 12). *IL12RB2* encodes an IL12 receptor chain. IL12 plays an important role in T-helper–1 responses, T-cell and NK-cell cytotoxicity, and interferon-γ (IFNγ) production by T cells and NK cells. IL12RB2 has been reported to be essential for high-affinity IL12 binding and IL12 dependent signaling, to be upregulated by interferon-γ (IFNγ) in Th1 cells and to have a crucial role in Th1 cell differentiation[13].

IL10 is a potent suppressor of inflammatory cytokines such as IL1, IL6, IL12, TNFα and IFNγ and inhibits the costimulatory activity of macrophages for T-cell and NK-cell activation. A previous study described three *IL10* promoter SNPs, rs1800896 (IL10-1082A/G), rs1800871 (IL10-819C/T) and rs1800872 (IL10-592A/C) (ref. 14), and recent independent studies have reported that *IL10* promoter SNPs were associated with several diseases. Further, another study has shown that the *IL10* promoter SNP rs1800871 was strongly associated with Behçet's disease[15].

We report here a GWAS identifying two new susceptibility loci for Behçet's disease; these loci include interleukin and interleukin receptor genes, which are central in immune response. The quantitative alteration of these cytokines (and others in the same cascade) could help explain in part the complex pathophysiology of Behçet's disease and suggest new therapeutic avenues.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

N.M. designed and supervised the experiment, provided study samples, performed data analysis and wrote the manuscript. A.M. designed the experiment, performed the SNP selection, supervised genotyping, performed data analysis, wrote the manuscript and prepared the tables and figures. M.O. participated in the experimental design, helped with data analysis and edited the manuscript. S.O. participated in the experimental design, provided study samples and edited the manuscript. T.S., T.K., N.I. and K.Y. performed genotyping. J.K. helped with data analysis. E.O., Y.W.S., E.B.L., N.K., K.N., Y.H., M.T., S.S., M.M. and Y.I. provided study samples. S.B. participated in the experimental design, helped with data analysis and participated in critical revisions of the manuscript. H.I. participated in the experimental design, provided study samples, helped with data analysis and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Kaklamani, V.G., Vaiopoulos, G. & Kaklamanis, P.G. Behcet's disease. *Semin. Arthritis Rheum.* **27**, 197–217 (1998).
2. Ohno, S. *et al.* Close association of HLA-Bw51 with Behcet's disease. *Arch. Ophthalmol.* **100**, 1455–1458 (1982).
3. Meguro, A. *et al.* Genetics of Behcet's disease inside and outside the MHC. *Ann. Rheum. Dis.* **69**, 747–754 (2010).
4. Fei, Y. *et al.* Identification of novel genetic susceptibility loci for Behcet's disease using a genome-wide association study. *Arthritis Res. Ther.* **11**, R66 (2009).
5. Remmers, E.F. *et al.* Genome-wide association study identifies variants in the MHC class I, *IL10*, and *IL23R-IL12RB2* regions associated with Behçet's disease. *Nat. Genet.* advance online publication, doi:10.1038/ng.625 (11 July 2010).
6. Duerr, R.H. *et al.* A genome-wide association study identifies *IL23R* as an inflammatory bowel disease gene. *Science* **314**, 1461–1463 (2006).
7. Cargill, M. *et al.* A large-scale genetic association study confirms *IL12B* and leads to the identification of *IL23R* as psoriasis-risk genes. *Am. J. Hum. Genet.* **80**, 273–290 (2007).
8. Liu, Y. *et al.* A genome-wide association study of psoriasis and psoriatic arthritis identifies new disease loci. *PLoS Genet.* **4**, e1000041 (2008).
9. Hirschfield, G.M. *et al.* Primary biliary cirrhosis associated with *HLA, IL12A*, and *IL12RB2* variants. *N. Engl. J. Med.* **360**, 2544–2555 (2009).
10. Rueda, B. *et al.* The *IL23R* Arg381Gln non-synonymous polymorphism confers susceptibility to ankylosing spondylitis. *Ann. Rheum. Dis.* **67**, 1451–1454 (2008).
11. Isomura, M. *et al. IL12RB2* and *ABCA1* genes are associated with susceptibility to radiation dermatitis. *Clin. Cancer Res.* **14**, 6683–6689 (2008).
12. Iwakura, Y. & Ishigame, H. The IL-23/IL-17 axis in inflammation. *J. Clin. Invest.* **116**, 1218–1222 (2006).
13. Chang, J.T., Shevach, E.M. & Segal, B.M. Regulation of interleukin (IL)-12 receptor beta2 subunit expression by endogenous IL-12: a critical step in the differentiation of pathogenic autoreactive T cells. *J. Exp. Med.* **189**, 969–978 (1999).
14. Turner, D.M. *et al.* An investigation of polymorphism in the interleukin-10 gene promoter. *Eur. J. Immunogenet.* **24**, 1–8 (1997).
15. Wallace, G.R. *et al.* IL-10 genotype analysis in patients with Behçet's disease. *Hum. Immunol.* **68**, 122–127 (2007).

ONLINE METHODS

Subjects. A total of 612 unrelated individuals with Behçet's disease and 740 unrelated healthy controls, all of Japanese descent, were enrolled in this study (**Supplementary Table 1**). The diagnosis of Behçet's disease was established according to standard criteria[16] proposed by the Japan Behçet's Disease Research Committee at the Yokohama City University, Hokkaido University, Tokyo Medical and Dental University, Kurume University, Yuasa Eye Clinic and Fujioka Eye Hospital. All control participants recruited from the Yokohama City University, Tokai University and Okada Eye Clinic were healthy volunteers unrelated to each other or to cases. Korean participants (119 cases and 140 healthy controls) were recruited at the Department of Internal Medicine, Seoul National University Hospital, South Korea. All cases were diagnosed according to criteria from the International Study Group for Behçet's disease (ISGBD)[17]. All control subjects were healthy Korean volunteers unrelated to each other or to cases. The study methodology complied with the guidelines of the Declaration of Helsinki. The study details were explained to all cases and controls before obtaining their consent for genetic screening. The QIAamp DNA Blood Maxi Kit (QIAGEN) was used to collect peripheral blood lymphocytes and extract genomic DNA from peripheral blood cells. Procedures were carried out under standardized conditions to prevent variation in DNA quality. Following extraction, DNA degradation and RNA contamination were assessed by 0.8% agarose gel electrophoresis.

Genotyping. *Genome-wide association study.* Genotyping with the GeneChip Human Mapping 500K Array Set (Affymetrix) was performed using the standard protocol recommended by Affymetrix. We set the standard for the minimum SNP call rate at 93% for each sample. The SNP call rates for all samples genotyped (612 cases and 740 controls) were ≥93%. To identify genotyping errors and DNA contaminations, we estimated the mean of genome-wide heterozygosity for all samples, and four samples (one case and three controls) that were more than 4 s.d. from the sample mean were excluded. Cryptic relatedness between samples was estimated by the identity-by-descent method using the PLINK software[18], and no samples were excluded by this analysis. This resulted in a dataset with 611 cases and 737 controls.

Quality control was performed on 500,568 SNPs from the GeneChip Human Mapping 500K Array Set, and we excluded 28,702 SNPs with a call rate < 95%, 14,044 SNPs with deviation from Hardy-Weinberg equilibrium in controls ($P < 0.001$) and 137,384 SNPs with a MAF < 5% overall. Therefore, 320,438 SNPs were left for subsequent analyses.

To assess possible population stratification in the GWAS samples, we carried out principal component analysis using EIGENSTRAT[19] in HelixTree, which showed no evidence of population admixture among the samples. To confirm the results of the principal component analysis, we estimated the genomic inflation factor λ based on the median of the χ^2 distribution and obtained an estimated value of $\lambda = 1.05$.

To exclude false-positive results, all SNPs showing significant evidence of association with Behçet's disease (defined as $P < 0.0001$) in the GWAS were assessed by clustering analysis; an association signal ($P < 0.01$) was found for a SNP in strong LD with a lead SNP (which had $P < 0.0001$). This lead SNP was considered to be a true-associated SNP. As a result, of the 83 SNPs with $P < 0.0001$ in non-*HLA* regions, 29 that clustered poorly were excluded from further analysis.

Fine mapping. We selected tagging SNPs covering the regions containing the genes encoding IL23R, IL12RB2 and IL10 from HapMap Japanese data (MAF ≥ 5%, pairwise $r^2 \geq 0.8$ and Hardy-Weinberg equilibrium $P \geq 0.05$) using the Haploview 4.1 software[20]. In addition, known non-synonymous SNPs and/or other disease-associated SNPs in the region containing the genes encoding IL23R, IL12RB2 and IL10 were also selected. Moreover, to cover the entire IL10 region more densely, two SNPs with MAF ~ 1% were selected in the region.

Genotyping of all SNPs was performed by TaqMan 5′ exonuclease assay using primers supplied by Applied Biosystems. The probe fluorescence signal was detected using the TaqMan Assay for Real-Time PCR (7900HT Sequence Detection System, Applied Biosystems) following the manufacturer's instructions.

Replication studies. We tested rs1495965, rs1800872 and rs1800871 using the same TaqMan assays for replication of 259 Korean individuals. We obtained the genotyping results in 2,494 Turkish individuals from colleagues performing a GWAS of Behçet's disease in Turkish individuals[5].

Statistical analyses. All association analyses for our GWAS were carried out using the software HelixTree SVS 7 (Golden Helix, Inc.). The association analyses for the fine-mapping stage were carried out using Haploview 4.1. Haploview 4.1 was also used to infer the LD structure of the targeted regions. The meta-analyses from the various populations were performed using the Mantel-Haenszel method.

16. Mizushima, Y. Recent research into Behçet's disease in Japan. *Int. J. Tissue React.* **10**, 59–65 (1998).
17. Criteria for diagnosis of Behçet's disease. International Study Group for Behçet's Disease. *Lancet* **335**, 1078–1080 (1990).
18. Purcell, S. *et al.* PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.* **81**, 559–575 (2007).
19. Price, A.L. *et al.* Principal components analysis corrects for stratification in genome-wide association studies. *Nat. Genet.* **38**, 904–909 (2006).
20. Barrett, J.C., Fry, B., Maller, J. & Daly, M.J. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* **21**, 263–265 (2005).

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LABORATORY INVESTIGATION

Elevation of Surfactant Protein D, a Pulmonary Disease Biomarker, in the Sera of Uveitis Patients with Sarcoidosis

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Abstract

Purpose: Surfactant protein D (SP-D) is found in the epithelial cells of multiple mucosal surfaces. It is commonly used to diagnose and screen for pulmonary diseases. In the present study, serum levels of SP-D were measured in patients with uveitis to ascertain whether SP-D is a clinically useful laboratory parameter to diagnose sarcoidosis.

Methods: Sera were obtained from 81 patients with sarcoidosis, 16 patients with Behçet disease, 40 patients with HLA-B27 associated uveitis, 50 patients with Vogt-Koyanagi-Harada (VKH) disease, and 33 healthy volunteers. Serum SP-D levels were quantified with an SP-D enzyme immunoassay kit.

Results: In the healthy control subjects, the average serum SP-D level was 39.70 ng/ml; in the uveitis patients with sarcoidosis, the mean serum SP-D level was 57.0 ng/ml, and in the uveitis patients with other etiologies the mean levels were 38.63 ng/ml for Behçet disease, 38.18 ng/ml for HLA-B27 associated uveitis, and 31.32 ng/ml for the VKH patients. The average serum SP-D levels of patients with sarcoidosis were significantly higher than those of patients with any other uveitis etiologies or healthy controls ($P < 0.01$).

Conclusions: SP-D may be a less invasive and less expensive laboratory examination for sarcoidosis screening. SP-D should be considered as a new laboratory parameter for the diagnosis of uveitis and sarcoidosis. **Jpn J Ophthalmol** 2010;54:81–84 © Japanese Ophthalmological Society 2010

Keywords: ACE, intraocular inflammation, KL-6, sarcoidosis, SP-D

Introduction

Sarcoidosis is a multisystem chronic inflammatory disorder of unknown etiology[1] in which approximately half of the patients develop ocular inflammation.[2–4] At present, the decision to treat patients is still based mainly on clinical features of worsening respiratory symptoms with assessment by radiography or lung function tests. Even when systemic manifestations are mild, ocular tissues are often severely inflamed.[5] Sarcoidosis is a Th1-mediated disease, and we have demonstrated that the serum levels of a macrophage migration inhibitory factor, secreted by activated T cells, are elevated in uveitis patients with sarcoidosis.[6,7] Recent reports have identified other immunological markers that may be indicators of sarcoidosis, such as upregulated intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 on macrophages, or the presence of inducible NO synthase in bronchoalveolar lavage fluid (BALF).[8] It is well known that angiotensin-converting enzyme (ACE) and lysozyme are helpful serum markers for the diagnosis of sarcoidosis. Although it is not very sensitive, ACE is one of the most useful markers, showing high specificity for sarcoidosis.[9] We have reported that serum KL-6, a MUC-1 mucin-like glycoprotein with a high molecular weight that is strongly expressed on type II alveolar pneumonocytes as

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well as on bronchiolar epithelial cells, is a clinically useful complement to ACE measurement as a laboratory parameter for the diagnosis and follow-up of sarcoidosis.[10,11]

Surfactant protein D (SP-D) is found in the epithelial cells of multiple mucosal surfaces, and is additionally present in endothelial cells and in serum, suggesting systemic effects of the protein. It has been demonstrated that either inflammatory diseases or the inflammatory cytokine tumor necrosis factor can induce serum SP-D levels.[12–15]

In the present study, the serum levels of SP-D were measured in patients with uveitis to ascertain whether SP-D might be a clinically useful laboratory parameter for ophthalmologists to diagnose sarcoidosis.

Subjects and Methods

After obtaining informed consent in accordance with the tenets of the Declaration of Helsinki, sera were obtained from 81 patients with sarcoidosis, 16 patients with Behçet disease, 40 patients with HLA-B27 associated uveitis, 50 patients with Vogt-Koyanagi-Harada (VKH) disease, and 33 healthy volunteers. Blood samples were collected during each patient's first medical examination at the Uveitis Survey Clinic of the Hokkaido University Hospital. None of the patients had received systemic treatment with corticosteroids or other immunosuppressive agents. The criteria established by the Diffuse Pulmonary Disease Research Committee of Japan were used for diagnosing patients with sarcoidosis. We chose histological results as the basis for this study. This study was approved by Institutional Review Board of Hokkaido University Hospital for Clinical Research.

Venous blood samples were collected in sterile vacuum tubes and centrifuged for 30 min as described previously.[6,16] Serum SP-D levels were quantified with an SP-D enzyme immunoassay kit from Yamasa Soy Sauce (Tokyo, Japan), which contains all necessary reagents for the assay. The results are presented as mean (standard error), and the statistical analysis of the SP-D levels was performed with the Student *t* test.

Results

The average level of serum SP-D in healthy control subjects was 39.70 (SE, 3.62) ng/ml. In uveitis patients with sarcoidosis, the mean serum SP-D level was 57.0 (4.12) ng/ml, and the uveitis patients with other etiologies had mean levels of 38.63 (5.19) ng/ml in Behçet disease; 38.18 (4.10) ng/ml in HLA-B27-associated uveitis; and 31.32 (2.70) ng/ml in VKH (Fig. 1). The serum levels of SP-D in sarcoidosis patients

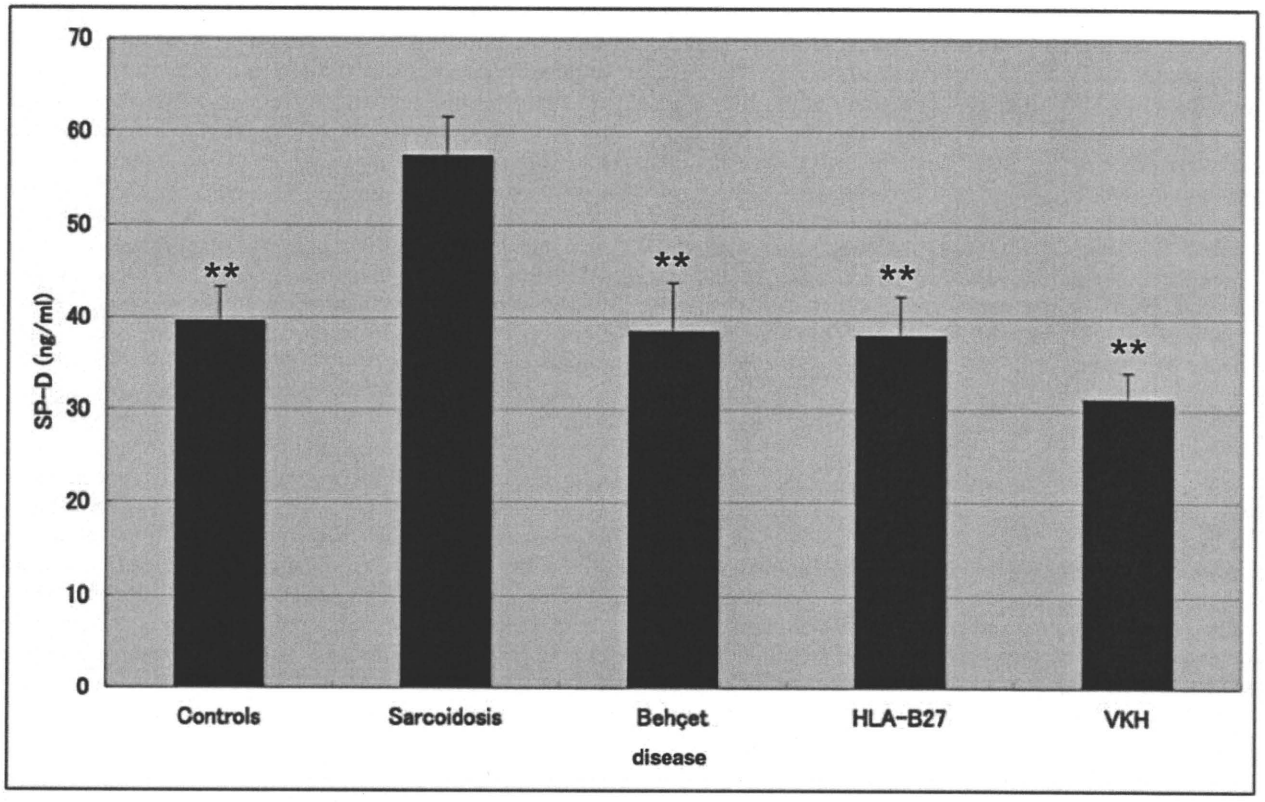

Figure 1. Serum surfactant protein D (*SP-D*) levels. Mean serum SP-D levels were significantly higher in patients with sarcoidosis than in those with Behçet disease, HLA-B27 associated uveitis, Vogt-Koyanagi-Harada (*VKH*) disease, or healthy control subjects. $^{**}P < 0.01$.

Table 1. Serum SP-D and ACE levels in sarcoidosis with uveitis ($n = 81$)

	ACE	
	Positive	Negative
SP-D		
Positive	6	6
Negative	45	24

SP-D, surfactant protein D; ACE, angiotensin-converting enzyme.

Table 2. Serum SP-D and ACE levels in other etiologies of uveitis ($n = 91$)

	ACE	
	Positive	Negative
SP-D		
Positive	0	1
Negative	1	89

were significantly higher than those in healthy controls ($P = 0.0017$), Behçet disease ($P = 0.007$), HLA-B27 associated uveitis ($P = 0.0013$), or VKH disease ($P = 0.0000005$).

Patients were considered to have a positive indication for sarcoidosis if their SP-D serum concentration exceeded 100 ng/ml. We found that, based on their serum SP-D levels, 14.8% (12/81) of uveitis patients with sarcoidosis proved positive (Table 1). All patients with high levels of SP-D had lung involvement. By this criterion, all healthy volunteers (100%) and 90 of the 91 (99%) patients with other etiologies were negative for sarcoidosis (Table 2). Using the standard ACE assay, 63.0% of the sarcoidosis patients were indicated as positive and 1% of uveitis patients with other etiologies were indicated as positive (false positives) (Table 2). When the results of the SP-D and ACE assay were combined, 70.4% of sarcoidosis patients were positive. None of the patients with other etiologies were double positive for both SP-D and ACE (no false positives) (Table 2).

Discussion

Sarcoidosis is one of the most frequent etiologies of uveitis in Japan.[17,18] The Japanese criteria for diagnosing sarcoidosis include histological and clinical data. To make a clinical diagnosis, it is necessary to have complete clinical pictures of two or more organs and positive results from six specific diagnostic examinations (bilateral hilar lymphadenopathy, elevated serum ACE, a negative tuberculin skin reaction, positive Ga scintigram, elevated CD4/CD8 ratio in BALF, and elevated serum/urinary Ca levels). However, serum ACE is not a sensitive indicator of sarcoidosis, making it difficult to clinically diagnose some patients with sarcoidosis, even if their granulomatous uveitis is typical for sarcoidosis. Clinicians already use serum ACE levels as one laboratory marker for sarcoidosis, but ACE measurements identify only half of the uveitis patients with sarcoidosis.[10] As it is thought that granulomatous tissue can release ACE, it is possible that the serum levels of ACE increase in other granulomatous conditions such as tuberculosis, lymphoma, and asbestosis. Serum ACE levels are affected by systemic corticosteroids.[11] In addition, some patients who are suffering from hypertension or heart failure may be treated with ACE inhibitors, which are antihypertensive drugs. In those patients, serum ACE levels often fall below measurable limits.[11]

We previously reported that MUC-1 mucin-like glycoprotein KL-6 levels were also elevated in sarcoidosis patients, and the sensitivity was raised to 87.5%, but there were 10.8% false positives when ACE and KL-6 serum measurements were combined.[10] However, as we later demonstrated, serum KL-6 levels are also elevated in patients with tubulointerstitial nephritis and uveitis (TINU) syndrome as well as sarcoidosis, because KL-6 is expressed in the renal distal tubes in addition to alveolar cells of the lungs.[19] Although it is not known whether the lung surfactant SP-D is expressed in renal tissues, it is unlikely to have a significant impact on serum levels in renal disorders. To examine the serum SP-D level in TINU syndrome may be a subject of future investigation.

Serum SP-D has been used as a prognostic or predictive marker for pulmonary inflammatory diseases (e.g., allergic bronchopulmonary aspergillosis, pneumonia, lung diseases with polymyosis, and acute lung injury).[20] Since SP-D is mainly synthesized by epithelial type II cells in pulmonary organs, the origin is supposed to be a result of leakage from SP-D-expressing tissues.[20] As some sarcoidosis patients suffer from damage to the lungs, it is reasonable to suggest that the elevated serum levels of SP-D in sarcoidosis are due to its release by pulmonary tissues. There is no report of SP-D expression in ocular tissues. Serum SP-D levels may reflect the involvement of pulmonary tissues in sarcoidosis but not ocular inflammation. High SP-D levels suggest the involvement of inflammation in another organ. In addition, because SP-D levels should be unaffected by ACE inhibitors, it may be worthwhile to add measurement of serum SP-D levels for sarcoidosis in patients with suspected uveitis.

Histological examinations of the lungs, bronchial tubes, and conjunctiva and lacrimal gland biopsy specimens are now accepted in diagnosing sarcoidosis. However, as biopsy sometimes causes much discomfort and pain, use of another laboratory parameter, SP-D, may be less invasive and less expensive when screening for sarcoidosis. SP-D should be considered as a new and useful laboratory parameter for the diagnosis of sarcoidosis and differential diagnosis of various uveitis entities.

References

1. Newman LS, Rose CS, Maier LA. Sarcoidosis. N Engl J Med 1997;336:1224–1234.

2. Crick RP, Hoyle C, Smellie H. The eyes in sarcoidosis. Br J Ophthalmol 1961;45:461–481.
3. Obenauf CD, Shaw HE, Sydnor CF, et al. Sarcoidosis and its ophthalmic manifestations. Am J Ophthalmol 1978;86:648–655.
4. Jabs DA, Johns CJ. Ocular involvement in chronic sarcoidosis. Am J Ophthalmol 1986;102:297–301.
5. Uyama M. Uveitis in sarcoidosis. Int Ophthalmol Clin 2002;42: 143–150.
6. Kitaichi N, Kotake S, Sasamoto Y, et al. Prominent increase of macrophage migration inhibitory factor in the sera of patients with uveitis. Invest Ophthalmol Vis Sci 1999;40:247–250.
7. Kotake S, Kitaichi N, Ohno S. Macrophage migration inhibitory factor in uveitis. Int Ophthalmol Clin 2002;42:99–103.
8. Semenzato G, Bortoli M, Agostini C. Applied clinical immunology in sarcoidosis. Curr Opin Pulm Med 2002;8:441–444.
9. Kawaguchi T, Hanada A, Horie S, et al. Evaluation of characteristic ocular signs and systemic investigations in ocular sarcoidosis patients. Jpn J Ophthalmol 2007;51:121–126.
10. Kitaichi N, Kotake S, Shibuya H, et al. Increase of KL-6 in sera of uveitis patients with sarcoidosis. Graefes Arch Clin Exp Ophthalmol 2003;241:879–883.
11. Kitaichi N, Ariga T, Kase S, et al. Usefulness of quantifying serum KL-6 levels in the follow-up of uveitic patients with sarcoidosis. Graefes Arch Clin Exp Ophthalmol 2006;244:433–437.
12. Akiyama J, Hoffman A, Brown C, et al. Tissue distribution of surfactant proteins A and D in the mouse. J Histochem Cytochem 2002;50:993–996.
13. Fujita M, Shannon JM, Ouchi H, et al. Serum surfactant protein D is increased in acute and chronic inflammation in mice. Cytokine 2005;31:25–33.
14. Leth-Larsen R, Nordenbaek C, Tornoe I, et al. Surfactant protein D (SP-D) serum levels in patients with community-acquired pneumonia small star, filled. Clin Immunol 2003;108:29–37.
15. Madsen J, Kliem A, Tornoe I, et al. Localization of lung surfactant protein D on mucosal surfaces in human tissues. J Immunol 2000;164:5866–5870.
16. Kitaichi N, Kotake S, Mizue Y, et al. Increase of macrophage migration inhibitory factor in sera of patients with iridocyclitis. Br J Ophthalmol 2000;84:1423–1425.
17. Goto H, Mochizuki M, Yamaki K, et al. Epidemiological survey of intraocular inflammation in Japan. Jpn J Ophthalmol 2007; 51:41–44.
18. Kitamei H, Kitaichi N, Namba K, et al. Clinical features of intraocular inflammation in Hokkaido, Japan. Acta Ophthalmol 2009; 87:424–428.
19. Kase S, Kitaichi N, Namba K, et al. Elevation of serum Krebs von den Lunge-6 levels in patients with tubulointerstitial nephritis and uveitis syndrome. Am J Kidney Dis 2006;48:935–941.
20. Sorensen GL, Hjelmborg JVB, Kyvik KO, et al. Genetic and environmental influences of surfactant protein D serum levels. Am J Physiol Lung Cell Mol Physiol 2006;290:1010–1017.