together, we propose that enhanced expression of AREG in BMMC and PMBC may play a pivotal role in the pathogenesis of RA.

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Src homology 2 domain-containing protein tyrosine phosphatase substrate 1 regulates the induction of Langerhans cell maturation

Atsushi Fukunaga[1], Hiroshi Nagai[1], Xijun Yu[1], Shuntaro Oniki[1], Hideki Okazawa [2], Sei-ichiro Motegi[2], Ryuji Suzuki[3], Nakayuki Honma[4], Takashi Matozaki[2], Chikako Nishigori[1] and Tatsuya Horikawa[1]

[1] Division of Dermatology, Department of Clinical Molecular Medicine, Kobe University Graduate School of Medicine, Kobe, Japan
[2] Biosignal Research Center, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Japan
[3] Department of Rheumatology and Clinical Immunology, Clinical Research Center for Allergy and Rheumatology, National Sagamihara Hospital, Sagamihara, Japan
[4] Pharmaceutical Research Laboratories, KIRIN Brewery Co., Ltd., Takasaki, Japan

Recently, we reported that Src homology 2 domain-containing protein tyrosine phosphatase substrate 1 (SHPS-1) plays an important role in the migration of Langerhans cells (LC). Here, we show that SHPS-1 is involved in the maturation of LC. Immunofluorescence analysis on epidermal sheets for I-A or CD86 revealed that LC maturation induced by 2,4-dinitro-1-fluorobenzene (DNFB) or by TNF-α was inhibited by pretreatment with an anti-SHPS-1 monoclonal antibody (mAb) or with CD47-Fc fusion protein, a ligand for SHPS-1. Further, FACS analysis demonstrated that I-A^+ LC that had emigrated from skin explants expressed CD80 or CD86, whereas CD47-Fc protein reduced $CD80^{high+}$ or $CD86^{high+}$ cells. CD47-Fc protein also reduced the up-regulation of surface CD80 or CD86 by LC remaining in the skin explants. In SHPS-1 mutant mice, we observed that the up-regulation of surface CD86 and CCR7 by LC induced by DNFB as well as that of surface CD80 and CD86 by LC in skin explants was attenuated. Finally, contact hypersensitivity (CHS) response was suppressed in SHPS-1 mutant mice and in wild-type mice treated with an anti-SHPS-1 mAb. These observations indicate that SHPS-1 plays an important role in the maturation of LC *ex vivo* and *in vivo*, and that SHPS-1-CD47 interaction may negatively regulate CHS.

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Introduction

DC are potent antigen-presenting cells that play a crucial role in the initiation of immune responses to pathogens. Langerhans cells (LC) are specialized immature DC in the skin that reside in the suprabasal layers of the epidermis. When LC encounter exogenous antigens, including haptens and microorganisms, they capture and process them to generate MHC/peptide complexes on their surface. LC migrate from the epidermis to draining lymphoid tissues in order to initiate naive T cells and to present the MHC/peptide complexes to

Correspondence: Dr. Tatsuya Horikawa, Division of Dermatology, Department of Clinical Molecular Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe, 650-0017Japan
Fax:+81-78-382-6149
e-mail: thorikaw@med.kobe-u.ac.jp

Abbreviations: CCR7: CC chemokine receptor 7 · **CHS:** contact hypersensitivity · **DNFB:** 2,4-dinitro-1-fluorobenzene · **LC:** Langerhans cell · **SHPS-1:** Src homology 2 domain-containing protein tyrosine phosphatase substrate 1 · **SIRP α1:** signal-regulatory protein α1

T cells with the necessary adhesion molecules and costimulatory signals [1–7]. During this migration, LC mature from a dormant into an activated functional state and down-regulate their endocytotic activity. The maturation of LC is characterized by increased expression of cell-surface molecules such as MHC class II, CD11c, CD40, CD80, CD86, CC chemokine receptor 7 (CCR7) and Langerin and by decreased expression of E-cadherin [7–13]. Recently, it has been shown that this functional maturation of LC is affected by various factors such as corticosteroids, hyaluronan, ultraviolet irradiation, prostaglandin E2-EP4 signaling, and ligation of E-cadherin or mycophenolate mofetil [11, 14–18].

One signal-regulatory protein, Src homology 2 domain-containing protein tyrosine phosphatase substrate 1 (SHPS-1) [19], is also known as signal-regulatory protein α1 (SIRP α1) [20], BIT (brain Ig-like molecule with tyrosine-based activation motifs) [21], P84 [22], MFR (macrophage fusion receptor) [23] and MyD-1 [24]. In mice, SHPS-1 is expressed in myeloid cells, including monocytes, macrophages and DC [25]. SHPS-1 is a transmembrane glycoprotein whose extracellular domain comprises three immunoglobulin-like domains with multiple N-linked glycosylation sites, while the cytoplasmic domain of SHPS-1 contains four tyrosine residues that form two ITIM [26]. SHPS-1 was initially discovered as a tyrosine-phosphorylated transmembrane protein that binds SHP-1 or SHP-2. IAP/CD47, an integrin-associated protein, has been identified as an extracellular ligand for SHPS-1 [27]. The CD47-SHPS-1 system has been shown to regulate a novel cell-cell communication system that is important in immunology and hematology. CD47 on red blood cells binds to SHPS-1 on macrophages, thereby inhibiting macrophage activation and phagocytosis [28, 29]. The interaction of SHPS-1 with CD47 negatively regulates cellular responsiveness during T cell activation and during the induction of antigen-specific cytotoxic T lymphocytes by DC and monocytes [24, 30]. Ligation of SHPS-1 prevents the functional maturation of immature DC and suppresses IL-12 production by mature DC [31], although it has not been elucidated whether the ligation of SHPS-1 on LC affects the maturational state of LC.

We recently demonstrated that LC/DC migration is significantly inhibited by the ligation of SHPS-1 and that LC migration is impaired in SHPS-1 mutant mice that lack the intracellular domain of SHPS-1 [32]. In this study, we focused on LC maturation and investigated whether that process is affected by the ligation of SHPS-1. We now demonstrate that LC maturation induced by haptens, TNF-α or a skin explant culture is significantly inhibited by the ligation of SHPS-1 with an anti-SHPS-1 mAb or with a CD47-Fc fusion protein. We further show that LC maturation induced by haptens or a skin explant culture is impaired in SHPS-1 mutant mice. Finally, we show that the contact hypersensitivity (CHS) response is markedly attenuated in mice treated with the anti-SHPS-1 mAb before sensitization or in SHPS-1 mutant mice.

Results

The ligation of SHPS-1 suppresses 2,4-dinitro-1-fluorobenzene-induced maturation of LC

The expression levels of I-A, CD40, the costimulatory molecules CD80 and CD86 and the DC marker CD11c are considered to reflect the maturational state of LC [7, 10, 12, 33]. It has been reported that application of a hapten, such as DNFB, up-regulates I-A [7, 8, 12] and elevates the expression of CD86 but not CD80 in the I-A$^+$ population of murine epidermal cells [14] or migrated dermal LC [34]. Similar to a previous report, we did not observe the expression of CD80 on LC *in situ* or following 2,4-dinitro-1-fluorobenzene (DNFB) painting on the skin. Therefore, we evaluated whether the ligation of SHPS-1 affects the surface expression of I-A or CD86 by immunofluorescence analysis of epidermal sheets. To evaluate the level of expression of surface molecules, we assessed the I-A$^+$ area in each cell by computer image analysis (NIH image) [35]. Application of DNFB markedly increased I-A expression, whereas *in vivo* pretreatment with an anti-SHPS-1 mAb (Fig. 1A) or with CD47-Fc protein (Fig. 1B) significantly reduced the up-regulation of I-A expression. Similarly, application of DNFB significantly elevated CD86 expression per I-A$^+$ cell, whereas *in vivo* pretreatment with CD47-Fc protein markedly inhibited up-regulation of CD86 expression (Fig. 1C). Representative images are shown in Fig. 1D. We could not observe CD80 expression on I-A$^+$ cells in the epidermis (data not shown).

The ligation of SHPS-1 suppresses LC maturation induced by TNF-α treatment

TNF-α has been reported to play an important role in the maturation of LC or DC *in vitro* [10, 17, 36]. Therefore we examined whether *in vivo* treatment with TNF-α affected the surface expression of I-A by immunofluorescence analysis of epidermal sheets. Intradermal injection of TNF-α (50 ng/mL) significantly increased I-A expression compared with injection of rat IgG (as a control). We then investigated whether ligation of SHPS-1 affected the TNF-α-induced maturation of LC. *In vivo* pretreatment with an anti-SHPS-1 mAb significantly inhibited the up-regulation of I-A expression of LC (Fig. 2A). Representative images are shown in Fig. 2B.

The ligation of SHPS-1 inhibits LC maturation induced by epidermal explant culture

Larsen *et al.* established a skin culture system, which uses murine ear skin to investigate the emigration and maturation of DC [37]. Since we wanted to investigate the maturation of LC rather than DC, we modified the method to use epidermal sheets rather than whole skin sheets. Interestingly, most cells emigrating into the medium during 48 h expressed both I-A and CD80 (Fig. 3A) or CD86 (Fig. 3B). The addition of CD47-Fc protein into the medium slightly but significantly reduced the percentage of I-A^+ $CD80^{high+}$ emigrated cells (Fig. 3A and B) and I-A^+ $CD86^{high+}$ emigrated cells (Fig. 3C and D). Immunofluorescence analysis revealed that I-A^+ cells remaining in the epidermal sheets 24 h after incubation expressed CD80 (Fig. 3E) and CD86 (Fig. 3F), while I-A^+ cells in the epidermal sheets before incubation hardly expressed CD80 or CD86 (data not shown). These observations ensure that the epidermal explant culture system is useful for investigating LC maturation. Immunofluorescence analysis of epidermal sheets clearly showed that treatment with CD47-Fc protein inhibited the up-regulation of CD80 (Fig. 3E) and CD86 expression (Fig. 3F). Up-regulation of CD205 expression in LC was also observed following explant culture (data not shown). The up-regulation of CD205 was inhibited by CD47-Fc (Fig. 3G).

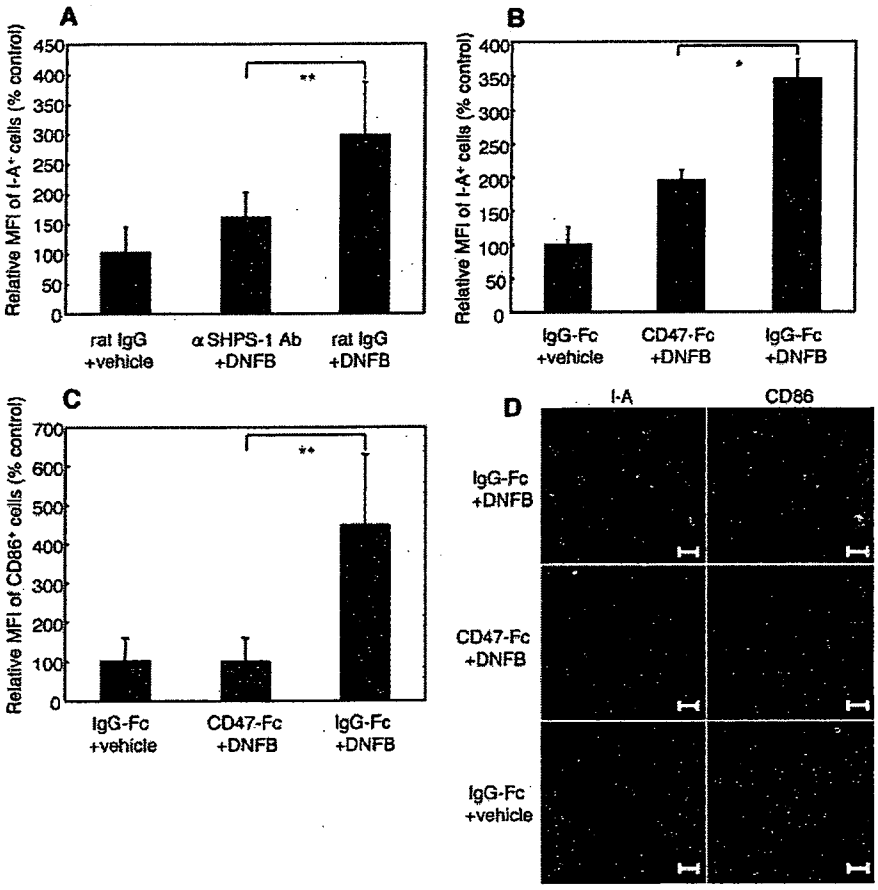


Figure 1. The ligation of SHPS-1 suppresses the DNFB-induced maturational state of LC. Anti-SHPS-1 mAb, CD47-Fc protein, or isotype-matched controls were intradermally injected before DNFB application. Twenty-four hours after the DNFB application, epidermal samples were collected and stained with FITC-conjugated anti-mouse $I\text{-}A^b$ mAb and PE-conjugated anti-mouse CD86 mAb. (A) Application of DNFB significantly increased the MFI of I-A expression, whereas the *in vivo* pretreatment of anti-SHPS-1 mAb significantly reduced the up-regulation of I-A expression. The ** indicates statistical significance compared with treatment of control antibody and DNFB ($p < 0.01$, $n = 4$). (B) CD47-Fc significantly reduced the up-regulation of I-A expression. The * indicates statistical significance compared with treatment of IgG-Fc and DNFB ($p < 0.05$, $n = 4$). (C) Application of DNFB significantly increased the MFI of CD86 expression, whereas *in vivo* pretreatment with CD47-Fc significantly reduced the up-regulation of CD86 expression. The ** indicates statistical significance compared with treatment of IgG-Fc and DNFB ($p < 0.01$, $n = 4$). (D) Representative images after treatment with CD47-Fc are shown. Bars indicate 20 μm.

LC maturation is attenuated in SHPS-1 mutant mice

Wild-type mice and mice with a mutation in SHPS-1 that lacks most of the cytoplasmic domain of the protein were painted with 0.5% DNFB and after 24 h epidermal samples were analyzed for expression of I-A$^+$ or CD86$^+$. CD86$^+$ cells were scarcely observed in the epidermis of SHPS-1 mutant mice or wild-type mice before DNFB application (data not shown). Application of DNFB remarkably increased the fluorescent intensity of CD86 on LC in wild-type mice, whereas CD86 expression after DNFB application was impaired in SHPS-1 mutant mice (Fig. 4A). Representative images are shown in Fig. 4B. Furthermore, application of DNFB remarkably increased the fluorescence intensity of I-A on LC in wild-type mice compared with SHPS-1 mutant mice (Fig. 4B). Epidermal explants from wild-type mice and from SHPS-1 mutant mice were incubated for 24 h and were then stained for I-A, CD80, or CD86 as described for Fig. 3E and F. Immunofluorescence analysis of epidermal sheets revealed that up-regulation of CD80 (Fig. 4C) and CD86 (Fig. 4D) was markedly inhibited in SHPS-1 mutant mice compared with wild-type mice.

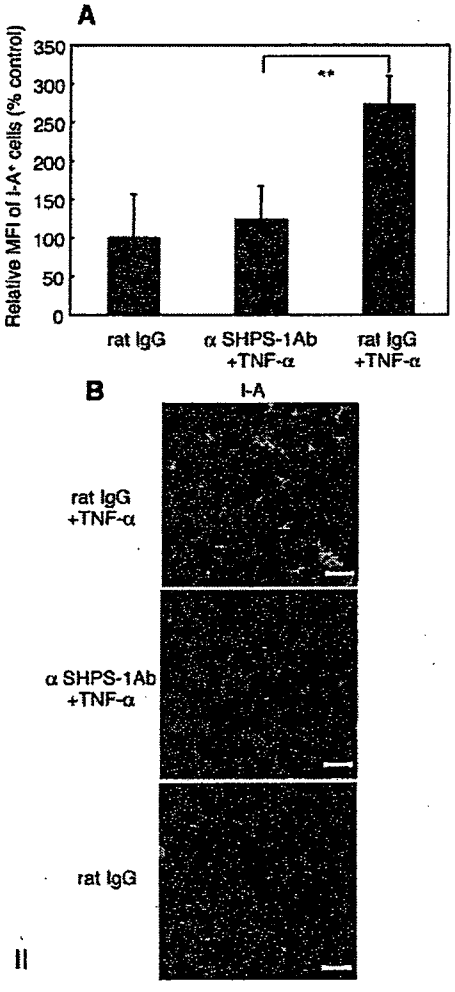


Figure 2. The ligation of SHPS-1 suppresses LC maturation induced by TNF-α- treatment. Anti-SHPS-1 mAb or rat IgG in combination with TNF-α was injected intradermally as described in the *Materials and methods*. One hour after the injection, epidermal samples were collected and stained for I-A. (A) Combined injection of rat IgG and TNF-α significantly increased the MFI of I-A expression, whereas combined injection of anti-SHPS-1 mAb and TNF-α significantly reduced the up-regulation of I-A expression. The ** indicates statistical significance compared with treatment of control antibody and TNF-α ($p < 0.01$, $n = 4$). (B) Representative images are shown. Bars indicate 20 μm.

Moreover, recent studies have shown that mature DC (but not immature DC) express CCR7, a chemokine receptor also known to regulate the entry of lymphocytes and LC into lymph nodes [38, 39]. Therefore, we further studied whether DNFB application affects the expression of CCR7 on LC residing in the epidermis. CCR7$^+$ cells were scarcely observed in the epidermis of SHPS-1 mutant mice or in wild-type mice before DNFB application (data not shown). The expression of CCR7 on I-A$^+$ cells in the epidermis of SHPS-1 mutant mice and of wild-type mice was hardly observed after application of vehicle (Fig. 5B). In contrast, application of DNFB increased the fluorescence intensity of CCR7 on LC in wild-type mice, whereas no increase of CCR7 expression was observed following DNFB application in SHPS-1 mutant mice (Fig. 5A and B).

The CHS response is attenuated in SHPS-1 mutant mice

To evaluate whether *in vivo* treatment with anti-SHPS-1 mAb influences the CHS response, we injected anti-SHPS-1 mAb intradermally before sensitization or elicitation. The CHS response to DNFB was significantly diminished by the treatment with anti-SHPS-1 mAb before sensitization (Fig. 6A). In contrast, the CHS response was not affected by the anti-SHPS-1 mAb treatment just before elicitation (data not shown). Furthermore, we assessed the ability to develop a CHS reaction in SHPS-1 mutant mice. The CHS response to DNFB was drastically decreased in SHPS-1 mutant mice compared with wild-type mice (Fig. 6B). We further examined the proliferation activity of regional lymph node cells in DNFB-sensitized mice. Addition of DNBS into the culture media induced marked proliferation of lymph node cells from wild type mice, while this proliferation was significantly impaired in SHPS-1 mutant mice (Fig. 6C). Similarly, DNBS stimulated the production of IFN-γ by lymph node cells in wild-type mice, whereas it was significantly reduced in SHPS-1 mutant mice (765 ± 262 *vs.* 133 ± 86 pg/mL; $n = 7$, $p < 0.05$).

Discussion

We showed in this study that ligation of SHPS-1 with an anti-SHPS-1 mAb or with CD47-Fc protein reduced the hapten-induced expression of I-A and CD86 by LC *in vivo*. Because expression of CD80 was hardly observed under the experimental conditions of DNFB painting on the skin *in vivo*, we studied the phenotypic maturational change of LC using a modified technique for the skin explant culture system [37]. As Hoetzenecker *et al.* [11] reported that expression by CD80 of LC in the epidermis was hardly observed in explant cultures of whole skin, we used the epidermis rather than the whole skin for skin explant culture [35]. That epidermal explant method enabled us to observe a significant induction of the expression of CD80 as well as of CD86 on LC remaining in the epidermis. Induction of the expression of those molecules was significantly inhibited by the addition of CD47-Fc protein. Thus, the epidermal skin explant culture system is useful for investigating the phenotypic maturation of LC by exogenous stimulants. We previously showed that SHPS-1 is selectively expressed on LC in the epidermis [32], which indicated that ligation of SHPS-1 with its ligands exclusively affects LC but not keratinocytes. These results indicate that engagement of SHPS-1 negatively regulates the phenotypic maturation of LC *in vivo* and *ex vivo*. We further observed that most cells migrating into the

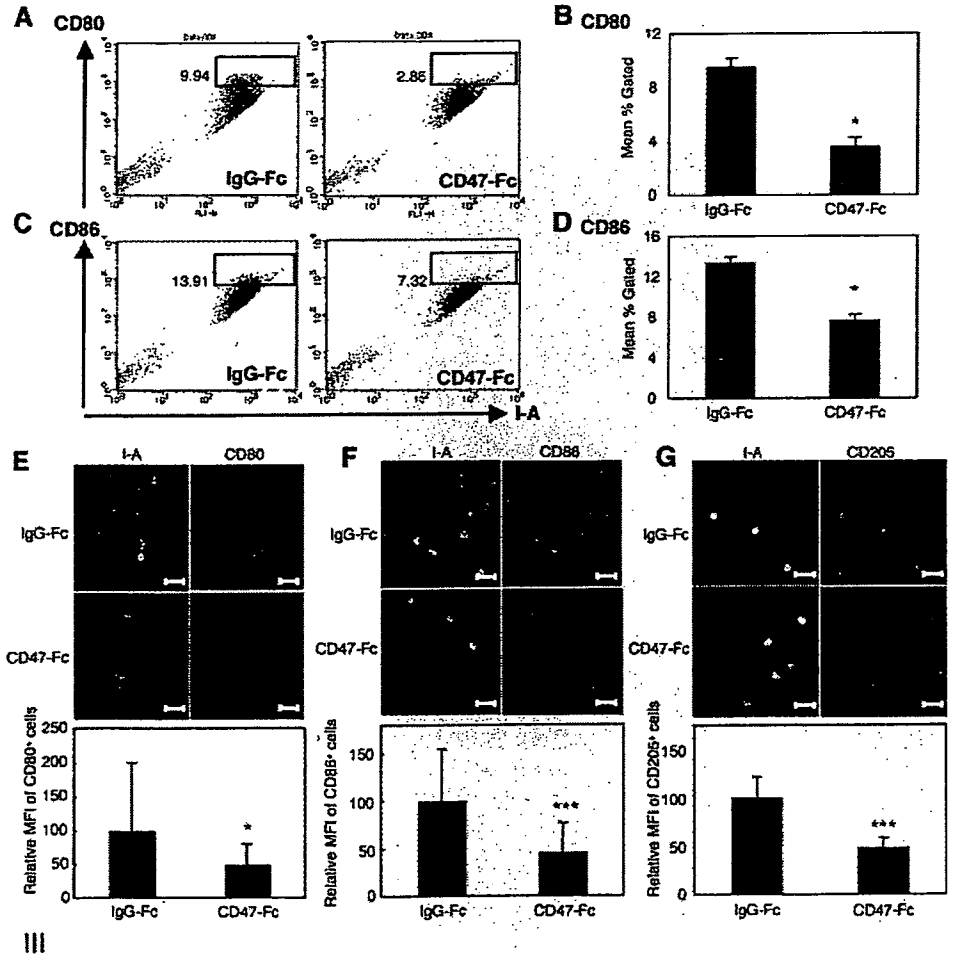
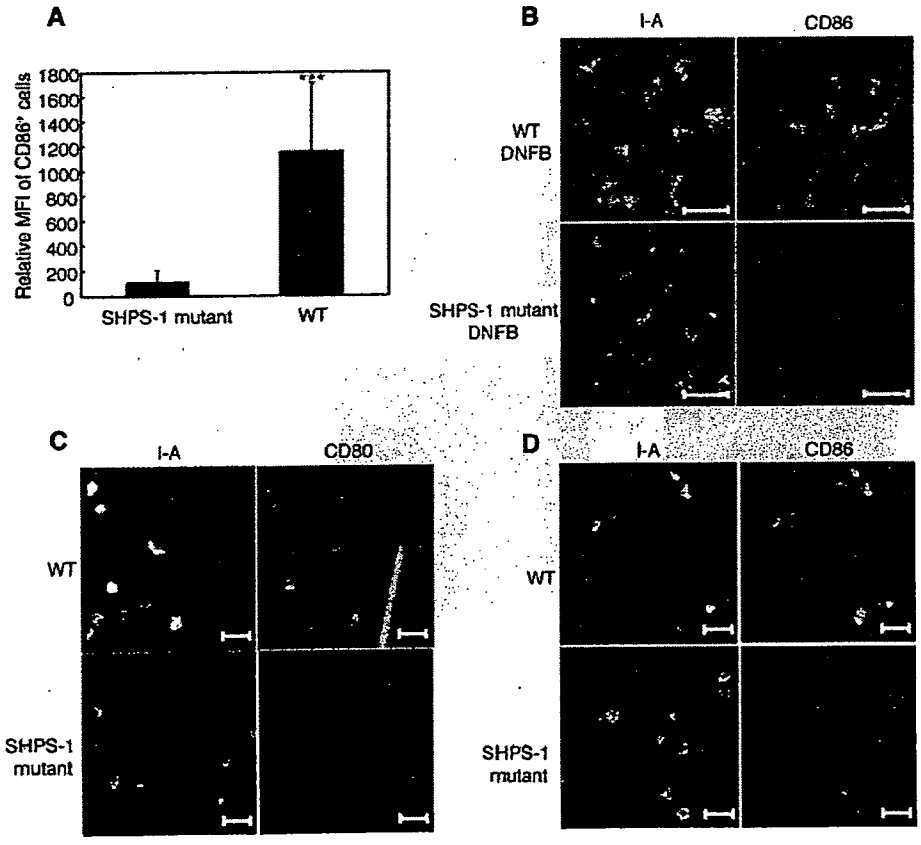


Figure 3. The ligation of SHPS-1 inhibits LC maturation induced by epidermal explant culture. The epidermal sheets were separated from the dermis by incubation of the dorsal halves of the ears in dispase. The epidermal sheets were cultured in 24-well tissue culture plates in 1.5 mL culture medium for 24 or 48 h at 37°C. (A, C) Cells that had emigrated into the culture medium during 48 h were evaluated by FACS. The emigrated cells typically expressed both I-A and CD80 or CD86. The addition of CD47-Fc protein into the medium obviously reduced the percentage of I-A$^+$ CD80^{high+} or I-A$^+$ CD86^{high+} emigrated cells, respectively. (B, D) Mean values of the percentage of I-A$^+$ CD80^{high+} or I-A$^+$ CD86^{high+} emigrated cells from three experiments are shown. Bars indicate SD. $^*p < 0.05$ (E, F, G) The epidermal sheets were collected after 24 h incubation and were stained for both I-A and either CD80, CD86 or CD205. The immunofluorescence analysis of epidermal sheets revealed that remaining I-A$^+$ cells in the epidermal sheets expressed CD80, CD86 and CD205, whereas additional treatment with CD47-Fc protein significantly inhibited the up-regulation of the expression. Bars in the photographs indicate 50 μm. $^*p < 0.05$, $^{***}p < 0.001$.

culture medium following epidermal explant culture expressed I-A, CD80 and CD86, indicating that the emigrated LC are in the maturation process. Ligation of SHPS-1 with CD47-Fc protein partially but significantly reduced the percentages of I-A$^+$ CD80^{high+} and I-A$^+$ CD86^{high+} cells in the emigrated LC population. The inhibitory effects of SHPS-1 ligation on maturation seem to be stronger in epidermal residing LC than emigrated cells. As it has been shown that binding of E-cadherin suppresses LC-like DC maturation [17], the reason why epidermal LC did not well maturate as compared to emigrated LC may be attributed to the homophilic binding of E-cadherin between LC and keratinocytes in the epidermis. We assume that ligation of SHPS-1 may thus cooperate with E-cadherin signals and greatly suppress LC maturation in the epidermis.

We further showed that the phenotypic maturation of LC by a hapten and *ex vivo* explant culture is suppressed in SHPS-1 mutant mice, which lack most of the intracellular domain of SHPS-1. Four tyrosine residues in the intracellular domain of SHPS-1 are believed to play a critical role in the transmission of the signals [20]. Phosphorylated tyrosine residues of SHPS-1 recruit the tyrosine phosphatases SHP-1 and SHP-2, which transmit the signals thereafter. The mutant protein in SHPS-1 mutant mice is not tyrosine phosphorylated, nor does it form a complex with SHP-1 or SHP-2 [40]. The SHPS-1 mutant mice have several characteristic features in the dysfunction of LC/DC and macrophages, including the motility of LC and the phagocytosis of macrophages. The finding that maturation of LC is attenuated in SHPS-1 mutant mice suggests that SHPS-1 is a prerequisite molecule for the LC functions including maturation.



IV

Figure 4. LC maturation induced by DNFB or by epidermal explant culture is attenuated in SHPS-1 mutant mice. SHPS-1 mutant mice and wild-type mice were painted with 0.5% DNFB on the ear and after 24 h the epidermal samples were analyzed for expression of I-A$^+$ or CD86$^+$ as described in legend of Fig. 1. (A) In wild-type mice, the MFI of CD86 expression increased after application of DNFB, whereas in SHPS-1 mutant mice, the up-regulation of CD86 expression was significantly suppressed. The *** indicates statistical significance compared with SHPS-1 mutant mice ($p < 0.001$, $n = 4$). (B) Representative images are shown. (C, D) Epidermal explants from wild-type mice and from SHPS-1 mutant mice were incubated for 24 h and were stained for I-A, CD80 or CD86. The up-regulation of CD80 (C) and CD86 (D) were obviously inhibited in SHPS-1 mutant mice compared with wild-type mice. Bars indicate 50 μm.

Especially, the phosphorylation of those four tyrosine residues in the intracellular domain of SHPS-1 and the consequent signal transduction is critical for LC maturation. Similarly, engagement of SHPS-1 has been shown to dephosphorylate the tyrosine residues in the intracellular domain of SHPS-1, resulting in the dissociation of SHP-2 [35]. In conjunction with the finding that ligation of SHPS-1 attenuates the maturation of LC, we assume that the maturation of LC requires the signal transduction *via* the phosphorylation of these tyrosine residues. This is further supported by the observation that the TNF-α-induced up-regulation of I-A expression by LC is attenuated by the ligation of SHPS-1, because it has been reported that TNF- α induces both the maturation of DC and the phosphorylation of tyrosine residues of SHPS-1 and the consequent recruitment of SHP-2 [10, 17].

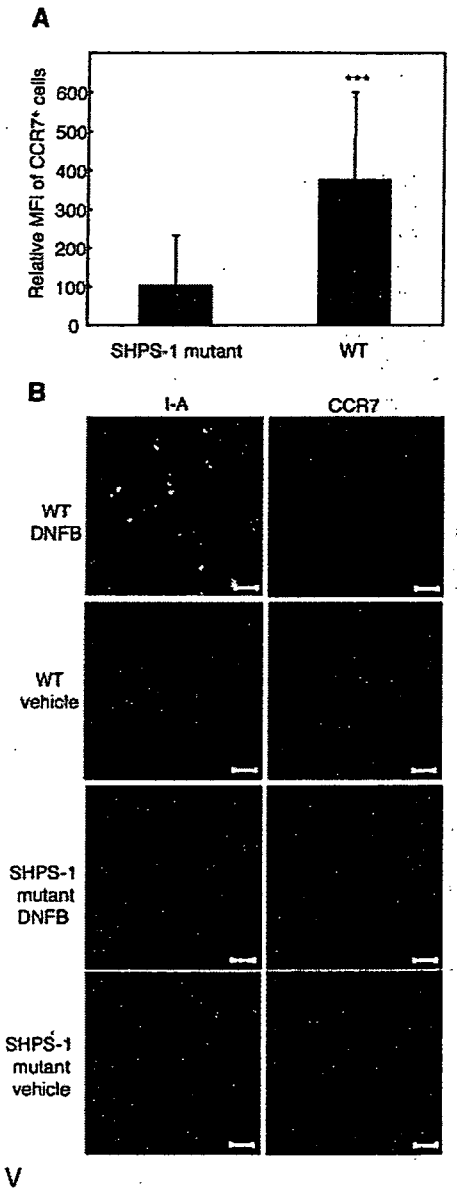


Figure 5. Up-regulation of CCR7 expression on LC induced by DNFB is attenuated in SHPS-1 mutant mice. (A) SHPS-1 mutant mice and wild-type mice were painted with 0.5% DNFB and after 24 h the ears were removed for analysis of I-A$^+$ or CCR7$^+$ LC. In wild-type mice, the MFI of CCR7 expression increased after application of DNFB, whereas in SHPS-1 mutant mice the up-regulation of CCR7 expression was significantly suppressed. The *** indicates statistical significance compared with SHPS-1 mutant mice (p <0.001, n = 6). (B) Representative images are shown. Bars indicate 20 μm.

We previously reported that the motility of LC is attenuated in wild-type mice by the engagement of SHPS-1 and in SHPS-1 mutant mice [32]. As immature DC, following induction of maturation after stimulation by inflammatory cytokines or haptens, up-regulate CCR7 [39, 41, 42], our next question was whether or not the expression of CCR7 on LC is impaired during the maturational changes following hapten application to SHPS-1 mutant mice. We found that expression of CCR7 on LC is suppressed following DNFB application in SHPS-1 mutant mice. Because expression of CCR7 on LC and its ligands, macrophage inflammatory protein-3β/CCL19 or a secondary lymphoid chemokine/CCL21 in lymph vessels and draining lymph nodes, is critical for the migration of LC from the epidermis into draining lymph nodes [38, 39], it is conceivable that the suppression of LC motility in SHPS-1 mutant mice might be attributed, at least in part, to the attenuated induction of CCR7. Because SHPS-1 is known to be involved in cell motility and adhesion, the inhibition of LC motility could be influenced both by the down-regulation of CCR7 expression and by the attenuated cell motility in SHPS-1 mutant mice.

We confirmed that the CHS response is suppressed in mice treated with SHPS-1 ligation before sensitization and in SHPS-1 mutant mice. Recently, three different groups reported three distinct results of CHS response by transient depletion of LC using mice that express diphtheria toxin receptor on LC; that is, (i) diminished (but not abrogated) [43], (ii) enhanced [44], and (iii) unchanged CHS response [45]. Thus, LC may not be the only cells that are required for the induction of CHS. Although we did not examine, it is plausible to consider that SHPS-1 ligation and its deficiency may affect maturation and motility of dermal DC, which may result in the diminished CHS response. The observation that the engagement of SHPS-1 inhibited the CHS response during the sensitization phase but not the elicitation phase suggests that the maturation and migration of LC/DC are possibly important for priming T cells but not for local inflammatory responses induced by antigen specific T cells. It has been shown that CD80 and CD86 play critical roles in the initiation of primary immune responses in the skin, in alloreactive immune responses and in antigen-specific T cell activation [34,

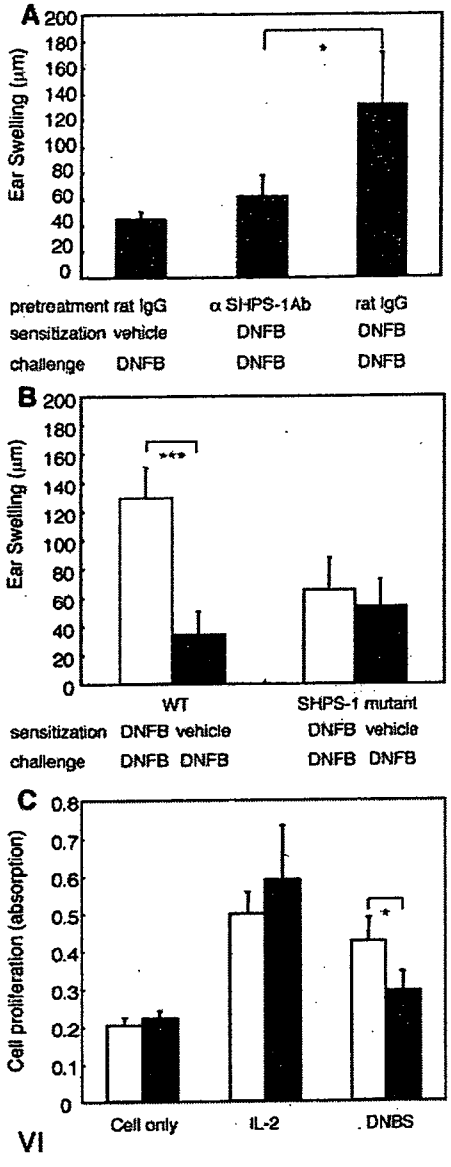


Figure 6. CHS responses are suppressed in mice treated with anti-SHPS-1 mAb before sensitization and in SHPS-1 mutant mice. The induction of the CHS reaction was conducted as described in the *Materials and methods*. CHS responses are expressed as the average increase of ear swelling with error bars representing SD for each group of six mice. (A) The CHS response to DNFB was significantly diminished by the treatment of anti-SHPS-1 mAb before sensitization compared with treatment of rat IgG. The * indicates statistical significance compared with the treatment of rat IgG and DNFB sensitization ($p < 0.05$, $n = 6$). (B) The CHS response to DNFB drastically decreased in SHPS-1 mutant mice compared with wild-type mice. The *** indicates statistical significance compared with the treatment of vehicle sensitization and DNFB challenge ($p < 0.001$, $n = 6$). (C) Five days after sensitization with DNFB, regional lymph nodes of the mice were collected and cultured with DNBS or IL-2, and cell proliferation was assessed as described in *Materials and methods*. Mean values of cell proliferation by DNBS were significantly impaired in SHPS-1 mutant mice, while those by IL-2 (50 U/mL) were unchanged. Representative data are shown from two separate experiments. The * indicates statistical significance compared with the SHPS-1 mutant mice and wild-type mice ($p < 0.05$, $n = 3$).

46]. We showed in this study that the expression of CD80 and CD86 was suppressed by SHPS-1 ligation. Thus, it is suggested that SHPS-1 plays important roles in the induction of both maturation and motility of LC and possibly of DC, which could be essential in the sensitization phase of CHS.

CD47, an SHPS-1 ligand, is widely expressed by a variety of cell types, including lymphocytes and endothelial cells [47]. Because keratinocytes express CD47 [35], maturation and migration might be negatively regulated by engagement with this molecule on cells surrounding LC *in situ*. We recently reported that LC also express CD47 of which ligation negatively regulates the maturation and motility of LC [35]. Thus, ligation of both SHPS-1 and CD47 on LC suppresses LC function, inhibiting priming T cells and consequent establishment of the immune response. It is possible that SHPS-1 and CD47 on LC bind each other or in a *cis* fashion.■ check sentence &block, So far, it is unclear whether the expression of SHPS-1 and CD47 is down-regulated during the maturational change and this is to be further studied.

Materials and methods

Mice

The generation of mutant mice that lack most of the cytoplasmic region of SHPS-1 has been previously described [48]. Mice were bred and maintained in the Institute of Experimental Animal Research of Gunma University or Kobe University Graduate School of Medicine under specific pathogen-free conditions. The mice were backcrossed onto the C57BL/6 background over five generations. Genotyping of the mice was performed by PCR analysis as previously reported [48]. Female C57BL/6 mice (6- to 8-week-old) were purchased from Charles River Japan (Tokyo, Japan). All animals were maintained in microisolator cages and exposed to a 12 h light/12 h dark cycle, with standard feed and water *ad libitum*. All animal experiments were conducted according to the Guidelines for Animal Experimentation at Kobe University Graduate School of Medicine or Gunma University.

Antibodies

Ascites were collected from BALB/c nu/nu mice that had been injected i.p. with the hybridoma producing rat anti-mouse-SHPS-1 mAb (p84) [49]. The p84 antibody was purified from the ascites using a protein A column. FITC- or PE-conjugated mouse anti-mouse I-A^b mAb (AF6–120.1) were purchased from BD Bioscience (Tokyo, Japan). PE-conjugated rat anti-mouse

CD80 mAb (RMMP2) and CD86 mAb (RMMP1) were purchased from Immunotech (Marseille, France). Rat anti-CD205 mAb was from UK-Serotec (Oxford, UK). Rat IgG was obtained from Sigma (St. Louis, MO). Goat polyclonal anti-CCR7 Ab and PE-conjugated donkey anti-goat IgG $F(ab')_2$ were purchased from Abcam (Cambridge, UK) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. CD47-Fc fusion protein was generated as previously described [50]. Briefly, CHO-Ras cells were transfected with pTracerCMV-hCD47-Fc. The CD47-Fc protein was then purified from the culture supernatant by column chromatography on Protein G HP (Amersham Pharmacia Biotech, Uppsala, Sweden). Human IgG-Fc fragment was obtained from Jackson Immunoresearch (West Grove, PA).

Preparation of murine epidermal sheets and immunofluorescence analysis

Murine epidermal sheets were prepared as previously described [32]. After fixation, the sheets were simultaneously incubated at room temperature for 30 min with FITC-conjugated mouse anti-mouse I-A^b mAb and PE-conjugated rat anti-mouse CD86 mAb, each diluted 1:100 in 5% BSA/PBS. In other experiments, the sheets were incubated at room temperature for 60 min with anti-mouse CCR7 Ab, washed with PBS, and thereafter was simultaneously incubated for 30 min with FITC-conjugated anti-mouse I-A^b mAb and PE-conjugated donkey anti-goat IgG $F(ab')_2$, each diluted 1:100 in 5% BSA/PBS. Finally, the sheets were washed with PBS and mounted on microscope slides in PermaFluor (Shandon, Pittsburgh, PA). The samples were analyzed using an Olympus Fluoview confocal laser scanning microscope (Olympus, Nagano, Japan). All specimens were scanned using the same emitting conditions. The relative MFI of I-A^b, CD86 or CCR7 were counted per one I-A^{b+} cell using NIH image. Similar experiments were made with SHPS-1 mutant mice.

LC maturation induced by DNFB application

Mice were painted with 10 μL 0.5% DNFB solubilized in acetone/olive oil (4/1) on both the dorsal and the ventral ear halves. Twenty-four hours later, the ears were collected for staining of LC as described above. To investigate the *in vivo* effects of anti-SHPS-1 mAb (p84), anti-SHPS-1 mAb or control rat IgG was injected intradermally at a dose of 60 μg/ear just before the DNFB application. Similarly, CD47-Fc protein or control human IgG-Fc was injected intradermally at 60 μg/ear just before the DNFB application.

LC maturation induced by TNF-α

Groups of mice (n = 5) received 30 μL intradermal injections of mouse rTNF-α (Sigma) (50 ng/ear) or an equivalent volume of carrier protein (0.1% bovine serum albumin) into the ear pinnae using 30-gauge stainless steel needles. Anti-SHPS-1 mAb or control rat IgG was injected intradermally at a dose of 60 μg/ear simultaneously. One h after the injection, the ears were collected for staining of LC as described above.

Skin organ culture

The epidermal sheets were prepared as previously described [32]. They were cultured in 24-well tissue culture plates in 1.5 mL culture medium (10% FCS, RPMI1640 with 20 μg/mL CD47-Fc protein or control human IgG-Fc) for 24 or 48 h at 37°C. At least six explants were cultured for each experimental condition. Cells that had emigrated from the epidermal skin explants into the culture medium during 48 h were evaluated phenotypically using a FACSCalibur flow cytometer and CELL QUEST (Becton Dickinson, San Jose, CA). In detail, cells that had emigrated were collected, washed in 1% BSA/PBS, and then were simultaneously incubated on ice for 30 min with FITC-conjugated mouse anti-mouse I-A^b mAb and PE-conjugated rat anti-mouse CD80 or CD86 mAb, each diluted 1:100 in 1% BSA/PBS. After a final washing, each sample was analyzed using a FACSCalibur flow cytometer and CELL QUEST. In another experiment, after 24 h the epidermal sheets were collected and fixed in acetone for 3 to 5 min at –20°C. After fixation, the sheets were washed in PBS and then were simultaneously incubated at room temperature for 30 min with FITC-conjugated mouse anti-mouse I-A^b mAb and PE-conjugated rat anti-mouse CD80, CD86 or CD205 mAb, each diluted 1:100 in 5% BSA/PBS. After washing with PBS, the sheets were mounted on microscope slides in PermaFluor. The samples were analyzed as described above.

CHS response and cell proliferation assay

Mice were sensitized by applying 25 μL DNFB solution (0.5% in acetone/olive oil, 4/1) on their shaved abdomens on day 0. On day 5, 10 μL 0.2% DNFB was applied to the dorsal and ventral aspects of the right ear, and the vehicle (acetone/olive oil) was applied to the left ear.

To investigate the *in vivo* effects of anti-SHPS-1 mAb, rat anti-SHPS-1 mAb or control rat IgG were injected intradermally in the right ear at a dose of 60 μg just before the DNFB application and 10 μL 1% DNFB or the vehicle (acetone/olive oil) was applied to the dorsal and ventral aspects of the right ear on day 0. On day 5, 10 μL 0.2% DNFB was applied to the dorsal and ventral aspects of the left ear. Ear swelling was measured in a blinded fashion with a digimatic micrometer (Mitutoyo, Kawasaki, Japan) 24 h after the challenge.

For cell proliferation assay, the regional lymph node cells were collected 5 days after DNFB sensitization. The cells (3×10^5) in 96-well plates were cultured with DNBS (Sigma, 30 μg/mL) in complete RPMI (RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 10 mM HEPES, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 5×10^{-5} M 2-ME (Nacalai Tesque, Tokyo, Japan), and 1% penicillin/streptomycin/ amphotericin B (Bio-Whittaker., Walkersville, MD)) for 3 days. For the last 4 h, each well was pulsed with MTS reagent [CellTiter® Aqueous One Solution Proliferation assay kit (Promega, Madison, WI)] and the proliferation activity of each well was measured using an ELISA reader (Nippon Bio-Rad Laboratories). Simultaneously, the supernatants after three days culture were collected and assessed the contents of IFN-γ using mouse IFN-γ ELISA kit (R&D System, Minneapolis, USA).

Statistical analysis

The statistical significance of differences between the means was determined using Student's *t*-test. Differences are considered statistically significant at $p < 0.05$. Each experiment was performed at least two times.

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Limited VH gene usage in B-cell clones established with nurse-like cells from patients with rheumatoid arthritis

S. Nakamura-Kikuoka[1], K. Takahi[2], H. Tsuboi[2], T. Toyosaki-Maeda[1], M. Maeda-Tanimura[1], C. Wakasa[1], N. Kikuchi[1], S. Norioka[3], M. Iwasaki[1], T. Matsutani[4], T. Itoh[4], S. Yamane[1], H. Takemoto[1], Y. Tsuruta[1], Y. Shimaoka[2], M. Yukioka[5], R. Suzuki[1,6] and T. Ochi[6]

Objectives. Nurse-like stromal cells (NLC) in synovia and bone marrow of patients with rheumatoid arthritis (RA) can support pseudoemperipolesis, protect from apoptosis and enhance immunoglobulin production of peripheral blood B cells isolated from healthy individuals, suggesting the profound contribution of hyperactivation of B cells in RA. In the course of establishing RA-NLC from RA patients, we observed the growth of B cells in the presence of RA-NLC.

Methods. We cloned B cells from the synovium or bone marrow of RA patients using the limiting dilution technique. For established clones, nucleotide sequences of immunoglobulin and surface antigens were investigated. To investigate the dependence of these clones on NLC, differences in the proliferation and the amount of immunoglobulin produced in the presence or absence of NLC were compared. Immunocytochemical staining of various cells was performed using the antibody these clones produced.

Results. Nine B-cell clones established from RA patients showed RA-NLC-dependent growth. These B-cell clones expressed CD19, CD20, CD38, CD39 and CD40, suggesting that the cloned cells were mature and activated. All clones secreted immunoglobulins in culture media, which were specific for intracellular components of various cell lines, including RA-NLC. Interestingly, we found limited usage of immunoglobulin heavy-chain variable regions (VH) among B-cell clones from RA patients. These repertoires were reported to be detected preferentially in fetal livers.

Conclusion. The present study provides a novel insight into the involvement of RA-NLC in the immunopathogenesis of RA via an autoreactive B cell development and/or activation mechanism.

KEY WORDS: Rheumatoid arthritis, B cell, Immunoglobulin VH gene, Autoantibody.

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by progressive joint destruction resulting from chronic inflammation [1]. Various inflammatory cells, including T cells, B cells and monocytes, infiltrate the synovium, and the formation of lymphonodular infiltrates containing lymphoid follicles is frequently seen [2]. Infiltrating B cells show several characteristics suggestive of an activated state: they proliferate and produce large amounts of immunoglobulin, such as rheumatoid factor (RF) [3]. RF is a group of autoantibodies that recognizes the constant region of IgG, and is frequently detected in patients with systemic autoimmune diseases [4]. On the other hand, various autoantibodies, including anti-nucleoprotein [5], anti-collagen [6, 7] and others [8–10] have been reported in the patients with RA. These findings suggest relationships between autoantibodies and autoimmune disease; however, B-cell involvement in these diseases is still to be elucidated.

Immunoglobulin heavy chain variable region (VH) gene of B cells in synovium of RA patients have been shown to be highly mutated, indicating an antigen-driven process of affinity maturation [11]. Although several studies showed that B-cell maturation occurred in affected joints [12–14], little is known about the precise mechanism. Reparon-Schuijt and colleagues [3] reported that fibroblast-like RA synoviocytes isolated from synovial fluid of RA patients stimulate IgM-RF production of B cells, suggesting the contribution of non-lymphoid cells in B-cell activation in RA joints.

We previously reported the establishment of nurse-like stromal cell lines (NLC) from bone marrow and synovium of RA patients [15, 16]. The RA-NLC as well as bone marrow stromal cells [17] supported the survival of B cells and enhanced their function [18]. NLC were not detected in the synovium of osteoarthritis patients or of normal subjects. These results suggested that RA-NLC may be a specific component of RA synovia and play an important role in RA pathogenesis.

In the present study, we characterized NLC-dependent B cells established from RA synovial tissue. The growth of B cells was

[1]Discovery Research Laboratories, Shionogi & Co., Ltd, [2]Department of Orthopaedics, Osaka University Medical School, [3]Graduate School of Frontier Biosciences, Osaka University, [5]Yukioka Hospital, Osaka, [4]Department of Cell Biology, Tohoku University School of Medicine, Sendai and [6]Clinical Research Center for Rheumatology and Allergy, National Sagamihara Hospital, Kanagawa, Japan.

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Correspondence to: T. Ochi, Clinical Research Center for Rheumatology and Allergy, National Sagamihara Hospital, 18-1 Sakuradai, Sagamihara, Kanagawa 228-8522, Japan. E-mail: t-ochi@sagamihara-hosp.gr.jp.

dependent on direct cell–cell contact with RA-NLC. These B cells secreted a large amount of immunoglobulin and were $CD5^-$, $CD19^+$, $CD20^+$, $CD38^+$, $CD39^+$. Biased usage of VH repertoires was observed in B-cell lines established from each individual. Similar limited VH repertoires were previously reported in the fetal liver B cells [19–21] and autoreactive B cells [22]. The evidence for selective B cell-activation in the presence of RA-NLC could provide new insights into the immunopathogenesis of RA.

Materials and methods

Patients and specimens

All specimens were obtained with consent and used in accordance with the policies and procedures of the research institutional review board for human subjects at each laboratory and hospital. Synovial tissues were obtained from five RA patients and a heparinized bone marrow aspirate was obtained from one RA patient. All patients in this study fulfilled the American College of Rheumatology Revised Criteria [23] for the diagnosis of RA at the time of joint reconstructive surgery in Osaka University Hospital.

Cell lines

HEp-2 (human epidermoid carcinoma), SiHa (human squamous carcinoma), Hs729 (human rhabdomyosarcoma) and ACHN (human adenocarcinoma) cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Gibco BRL) and antibiotics (complete DMEM) in 7.5% CO_2 at 37°C. RA-NLC were established from primary cultures of synovial tissues and bone marrow of RA patients as described previously [16, 18]. Briefly, synovial tissues were teased apart with scissors and digested into single cells using collagenase and hyaluronidase. The cells were washed and cultured in complete DMEM in 7.5% CO_2 at 37°C. Heparinized bone marrow was obtained from the iliac crest. Mononuclear cells were separated by Ficoll–Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation and the cell suspensions were cultured in complete DMEM for 2 weeks. After non-adherent cells had been removed, adherent cells were maintained in complete DMEM in 7.5% CO_2 at 37°C.

Establishment of RA-NLC-dependent B-cell lines and clones

To establish RA-NLC, primary cultures of the synovial tissue or the bone marrow were maintained over 6 months allowing the proliferation of lymphoblastic cells. The cells were adjusted to $2–3 \times 10^5$ in 75 cm^2 culture flasks (Costar, Corning, NY, USA) every week. After lymphoblastic cells were sufficiently grown, these cells were stained for B-cell surface antigens as described below. B-cell clones were established from these B-cell lines by the limiting dilution method. Briefly, ten thousand autologous RA-NLC were cultured in 96-well plate (Costar) for 3 days, then B-cell lines were cocultured on RA-NLC at 0.5 or 1 cell per well in complete DMEM. Cells, when proliferated, were transferred into a 48-well culture plate (Becton Dickinson Labware, Bedford, MA, USA) and further expanded with autologous RA-NLC. Since no significant difference was seen in the phenotypes and immunoglobulin production of B cells cocultured with autologous RA-NLC from those with allogenic RA-NLC (data not shown), clones were maintained either with autologous or allogenic RA-NLC every month.

Molecular analysis of immunoglobulin genes

Immunoglobulin heavy chain genes of each B cell were amplified by adapter-ligation mediated PCR as described previously [24] with minor modification. Briefly, total RNA from each of the B cells isolated using Trizol reagent (Gibco BRL) were submitted for synthesis of first-strand cDNA using BSL-18 primer, which contains oligo-dT and the *Not*I cutting site. After second-strand synthesis, P20EA/P10EA adapter was ligated to double-stranded cDNA, then digested with *Not*I. Adapter-ligated cDNAs were submitted for nested PCR using P20EA and primers specific for C-regions. The sequences of PCR primers were as follows: IgG, first PCR (CG1: CAC CTT GGT GTT GCT GGG CTT), second PCR (CG2: TCC TGA GGA CTG TAG GAC AGC); IgA, first PCR (CA1: GCT GGC TGC TCG TGG TGT AC), second PCR (CA2: GGG AAG TTT CTG GCG GTC ACG); IgM, 1st PCR (CM1: TCC TGT GCG AGG CAG CCA A), second PCR (CM2: GTA TCC GAC GGG GAA TTC TC).

Nested PCR products were cloned into pGEM-T vector (Promega, Madison, WI, USA). Dye terminator cycle sequencing was carried out using T7 primer according to the manufacturer's instruction (Beckman Coulter, Fullerton, CA, USA). The cDNA sequences were compared with the corresponding human germ-line VH gene segments in GenBank using the BLAST program.

Cell proliferation assay

RA-NLC (5×10^4cells/well) were cultured for 2 days in a 24-well flat-bottomed culture plate and treated with mitomycin C (MMC; Kyowa Hakko Kogyo, Tokyo, Japan) at 25 μg/ml at 37°C for 1 h. Cells were washed and seeded onto a 24-well flat-bottomed culture plate (Costar). The next day, 100 000 B-cell clones were cocultured with MMC-treated RA-NLC for 9 days. Cells were then pulsed with 0.5 μCi of tritiated thymidine (Radiochemical Center, Amersham, UK) for 18 h and were harvested onto glass filters. Radioactivity was measured on a β-scintillation counter. Experiments were performed with no additional cytokine or growth factor.

Measurement of the amount of immunoglobulin production

To determine the immunoglobulin isotype, culture supernatants of each B-cell clone were tested with a human immunoglobulin isotyping kit (The Binding Site, Birmingham, UK) based on the Ouchterlony immunodiffusion technique. B cells (1×10^5 cells) were cocultured with RA-NLC (5×10^4cells/well) which had been previously cultured for 2 days in a 24-well flat-bottomed culture plate. In some experiments, B cells were cultured on Millicell culture plate inserts (Nihon Millipore Kogyo, Yonezawa, Japan) to prevent direct contact with RA-NLC. On day 3, the culture supernatants were collected and the concentration of immunoglobulins was measured with the Human Immunoglobulin ELISA Quantitation Kit (Bethyl, Montgomery, TX, USA).

Purification of antibody from culture media

The antibodies were purified using protein A and/or protein G Sepharose 4B (Amersham Pharmacia Biotech) chromatography according to the manufacturer's instructions. For the detection of RF, purified antibodies were incubated with denatured human IgG-coated latex particles using total RA Test-N (Nissui Pharmaceutical, Tokyo, Japan).

Immunocytochemical staining

RA-NLC, HEp-2, SiHa, Hs729 and ACHN cells were cultured in a chamber slide (Nalge-Nunc International, Roskilde, Denmark)

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at 37°C. After 16 h, culture medium was removed and cells were fixed on the slides with cold acetone at −20°C. These slides were air-dried and stored at −80°C until use. For immunocytochemical staining, slides were incubated with purified antibodies at 100 μg/ml with 0.1% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) at 4°C for 18 h. Slides were washed with PBS, then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-human immunoglobulin antibody (Dako, Carpinteria, CA, USA) diluted 1:200 with 0.1% BSA/PBS for 2 h at 37°C. After washing with PBS, the slide was covered with a coverglass in 50% glycerol/PBS and examined under a fluorescence microscope.

Phenotype of B-cell clones

The clones were stained with FITC-conjugated anti-human monoclonal antibodies specific for CD5, CD11a, CD20, CD38 and CD40 (BD Pharmingen, San Diego, CA) and for human IgA, IgG, IgM, IgD, kappa and lambda (Dako). They were also stained with phycoerythrin-conjugated anti-human monoclonal antibodies specific for CD19 and CD39 (BD Pharmingen). Anti-human monoclonal antibodies specific for CD49d (Upstate Biotechnology, Lake Placid, NY, USA) was non-labelled and FITC-conjugated goat anti-mouse IgG (BD Pharmingen) was used as the secondary antibody. Anti-human CXCR4 (R&D Systems, Minneapolis, MN, USA) was detected by staining with Avidin-R-Phycoerythrin (Serotec, Raleigh, NC, USA). The stained cells were analysed using a FACScan™ (BD Pharmingen) flow cytometer and CellQuest software (BD Pharmingen). Dead cells were excluded by propidium iodide staining. Forward and side scatter gates were determined for lymphoblastic cells to exclude possible contamination by RA-NLC.

Detection of Epstein–Barr virus (EBV) genome

Genomic DNA samples were isolated from each B-cell clone using the DNeasy™ Tissue Kit (Qiagen, Valencia, CA, USA). To remove RA-NLC, B-cell clones were collected, washed and stained with CD19 antibody conjugated with magnetic microbeads (Miltenyi Biotec, Germany) and passed through the magnetic column. $CD19^+$ purity of the B-cell clones after the procedure was >98%, using FACS analysis. A PCR specific for the *Bam*HI W repeat region of the EBV genome was performed. Primers for the above region were synthesized and the PCR reaction was conducted as described previously [25]. Amplified products were electrophoresed in 1% agarose gel and visualized with ethidium bromide staining.

Western blot analysis of B-cell clones

The cell lysates were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) under reduced conditions and the electrophoresed protein was transferred to PVDF membrane (Millipore, Bedford, MA, USA) in a semi-dry blotting system. The membrane was blocked with 3% skim milk/1% BSA/PBS at 4°C for 18 h and then cut into strips and incubated with antibodies diluted with 1% BSA/PBS at room temperature for 3 h. Strips were washed with 0.5% Tween 20/PBS and reacted with horseradish peroxidase-labelled anti human immunoglobulin antibodies diluted 1:2000 with 0.1% BSA/PBS for 1 h. After washing, bound antibodies were detected with an electrochemiluminescence system (ECL; Amersham Pharmacia Biotech).

Immunoprecipitation

RA-NLC, HEp-2, SiHa, Hs729 and ACHN were cultured in semiconfluent conditions. Cells were collected with a cell scraper (BD Labware) and lysed with RIPA buffer (1% NP-40, 0.5% sodium deoxycolate, 0.1% SDS, 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 μg/ml aprotinin, 1 mM PMSF) or hexadecyltrimethyl-ammonium bromide (CTAB) buffer (1% CTAB, 5 mM EDTA, 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 μg/ml aprotinin, 1 mM PMSF). Cell lysates were mixed with purified antibodies at 4°C for 18 h. The immune complexes were recovered by protein G Sepharose 4B and the eluates were subjected to SDS–PAGE. The reacted proteins were visualized by silver staining (Wako Pure Chemical, Osaka, Japan).

Results

Establishment of B-cell clones and molecular analysis of immunoglobulin repertoire

We found that B cells proliferated in a long-term culture with NLC from RA bone marrow and synovial tissue. We analysed the immunoglobulin VH gene repertoire of these B-cell lines (Table 1). Since only one VH gene was detected in RA3 and RA45 cell lines, these lines were considered to be monoclonal. The B-cell lines generated from RA32 and RA79 had broader repertoires. Highly limited VH repertoires have been reported in B cells of the human fetal liver [19–21]. Interestingly, the majority of the detected VH gene usage of our B-cell lines was contained within the limited repertoire found in the human fetal liver.

Using the limiting dilution method, we established B-cell clones and analysed their immunoglobulin repertoire (Table 2) For clones established from RA3, RA32, RA45, RA79 and RA176, nucleotide sequences of the V-D-J region were all identical in each patient. In RA32 and RA79, broader repertoires were detected before cloning and only the most frequently used repertoire in each B-cell line was cloned. From RA133, four different B-cell clones were detected, with the VH1-69 gene subgroup used most frequently. Nucleotide sequences of clones from RA79 and RA176 belong to the same putative germ-line gene, 3-30, and RA3 and RA133 belong to the same germ-line gene, 3-48.

The replacement/silent (R/S) ratios in mutations of complementarity-determining region (CDR) of VH gene differed for every clone. All mutations were R in RA32; on the other hand, the number of mutations of R and S was the same in RA79. Moreover,

TABLE 1. Repertoire of immunoglobulin heavy-chain variable regions of B-cell lines established from RA patients

Patient	Origin	VH gene[a]	Homology[b] (%)	Frequency[c] (N/T)
RA3	Synovial tissue	3–48	95.9	9/9
RA32	Bone marrow	3–13	96.6	6/10
		3–21	95.6	2/10
		4–4	94.9	1/10
		4–4	93.2	1/10
RA45	Synovial tissue	3–9	94.2	6/6
RA79	Synovial tissue	3–30	93.8	13/36
		3–7	93.9	9/36
		2–5	87.9	8/36
		3–9	91.5	3/36
		3–7	89.1	1/36
		3–33	85.7	1/36
		4–18	86.8	1/36

[a]Bold face signifies VH usage corresponding to that previously reported in B cells in fetal liver [19–21]. Underlined values correspond to VH usage previously reported in autoantibodies [22]. [b]Homology was calculated by similarity at the nucleic acid level. [c]Number of immunoglobulin genes detected (N) out of total number (T) of sequences analysed.

TABLE 2. Analysis of immunoglobulin heavy-chain regions of the B-cell clones

Patient	Origin[a]	Representative name of clone	V gene subgroup	VH gene[b]	Homology[c] %	Homology[c] D gene subgroup	Homology[c] J gene subgroup	Frequency[d] (N/T)
RA3	Sy	RA3a	VH3	3–48	95.9	DH5	JH4	9/9
RA32	BM	RA32a	VH3	3–13	96.6	DH6	JH4	6/6
RA45	Sy	RA45a	VH3	3–9	94.2	DH6	JH4	6/6
RA79	Sy	RA79a	VH3	3–30	93.8	DH3	JH4	7/7
RA133	Sy	RA133a	VH1	1–69	95.9	DH3	JH4	4/10
	Sy	RA133b	VH3	3–66	91.4	DH6	JH5	3/10
	Sy	RA133c	VH3	3–48	89.0	DH2	JH3	2/10
	Sy	RA133d	VH5	5–51	91.5	DH3	JH3	1/10
RA176	Sy	RA176	VH3	3–30	92.1	DH5	JH4	6/6

[a]BM, bone marrow; Sy, synovial tissue. [b]Bold face signifies VH usage corresponding to that previously reported in B cells in fetal liver [19–21]. Underlined values correspond to VH usage previously reported in autoantibodies [22]. [c]Homology was calculated by similarity at the nucleic acid level. [d]Number of immunoglobulin genes detected (N) out of total number (T) of analysed sequences.

TABLE 3. Analysis of immunoglobulin heavy-chain regions of the B-cell clones

Name of clone	FR[a] R[b]	FR[a] S[b]	FR[a] R/S	CDR[a] R[b]	CDR[a] S[b]	CDR[a] R/S
RA3a	4	1	4.0	4	1	4.0
RA32a	4	1	4.0	5	0	–
RA45a	5	3	1.7	5	2	2.5
RA79a	7	1	7.0	4	4	1.0

[a]FR, framework; CDR, complementarity-determining region; [b]replacement (R) and silent (S) mutations in the framework and CDR regions.

comparison of the R/S ratio in the framework region (FR) and the CDR showed that every clone was different (Table 3).

In total, nine B-cell clones were established from six patients. Clones predominantly expressed members of VH3 family with no representation of the VH2 or VH4 family. The homology of VH regions of B-cell clones ranged from 89.0 to 96.6%. For JH regions, dominant usage of the JH4 family was observed, but there was no correlation between VH, JH and DH usage.

Nucleotide and amino acid sequences of CDR3 region are shown in Table 6 as supplementary data.

Immunoglobulin production of B-cell clones

The growth of B-cell clones was found to be RA-NLC-dependent, similar to previous findings in B-cell lines established from RA patients [16]. Direct contact with RA-NLC appeared necessary for the proliferation of B-cell clones. B cells showed no growth when cultured in medium alone, while proliferation was remarkably high in the presence of RA-NLC (Fig. 1).

To determine the isotype of secreted antibodies produced by these B-cell clones, culture supernatants were examined. B-cell clones predominantly produced IgG (7/9) while a minority produced either IgA (1/9) or IgM (1/9) (Table 4). The production of ~~Ig~~ was also observed only when cocultured with RA-NLC. If B-cell clones were cocultured with RA-NLC but separated by a cell culture insert, proliferation and immunoglobulin production were markedly down-regulated (Fig. 1, Table 5). In summary, the proliferation of B-cell clones and their production of immunoglobulin are dependent upon direct contact with RA-NLC.

immunoglobulin

Immunocytochemical staining with antibodies produced by B-cell clones

B-cell clones from our RA patients produced 10–50 mg/l of immunoglobulins in their culture supernatants (data not shown).

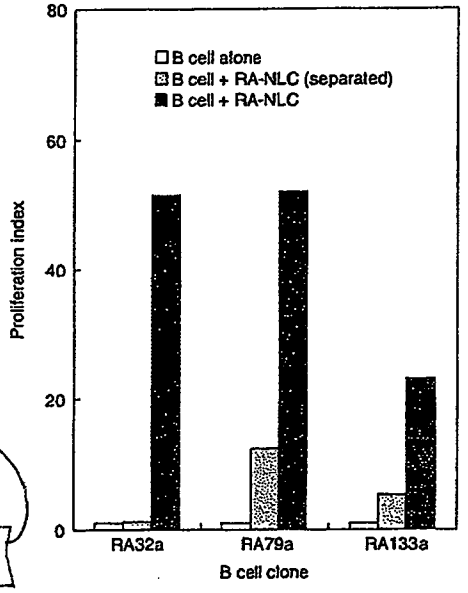

FIG. 1. Proliferation of established B-cell clones. B cells (1×10^5) were cultured alone or cocultured with or without direct contact with mitomycin C-treated RA-NLC (5×10^4) for 10 days. Proliferation index was calculated as experimental ^{3}H-TdR incorporation (mean c.p.m.)/^{3}H-TdR incorporation of B cells cultured alone (mean c.p.m.).

TABLE 4. Recognition of autoantigen by immunoglobulins produced by B-cell clones

Patient	Representative name of clone	Isotype[a]	Secreted immunoglobulin[b]	Immunofluorescence staining pattern
RA3	RA3a	μ/m/k/κ	IgM	NT
RA32	RA32a	γ/g/k/κ	IgG	nucleus (speckled)
RA45	RA45a	α/a/l/λ	IgA	Cytoplasm
RA79	RA79a	γ/g/k/κ	IgG	Cytoplasm
RA133	RA133a	γ/g/k/κ	IgG	Cytoplasm
	RA133b	γ/g/k/κ	IgG	Cytoplasm
	RA133c	γ/g/k/κ	IgG	NT
	RA133d	γ/g/k/κ	IgG	NT
RA176	RA176	γ/g/k/κ	IgG	Cytoplasm

[a]Determined by FACS analysis; [b]determined by Ouchterlony immunodiffusion method with culture supernatants. NT, not tested.

in Greek alphabet, if you can

TABLE 5. Immunoglobulin production by the B-cell clones

Culture condition	Immunoglobulin production[a] (ng/ml) RA32a	RA45a	RA79a
RA-NLC	<31.3[c]	<31.3	<31.3
B cells	49.1	33.8	<31.3
B cells + RA-NLC (separated)[b]	119.4	<31.3	<31.3
B cells + RA-NLC	150.2	>1000	>1000

B-cell clones (1×10^5) and NLC (5×10^4) were cultured for 3 days in 24-well plates. [a]The amount of immunoglobulin in the cell culture supernatants was measured with an ELISA kit as described in Materials and methods; [b]B-cell clones were cultured on a Millicell culture insert and RA-NLC were cultured on the bottom of the same well separately; [c]undetectable level.

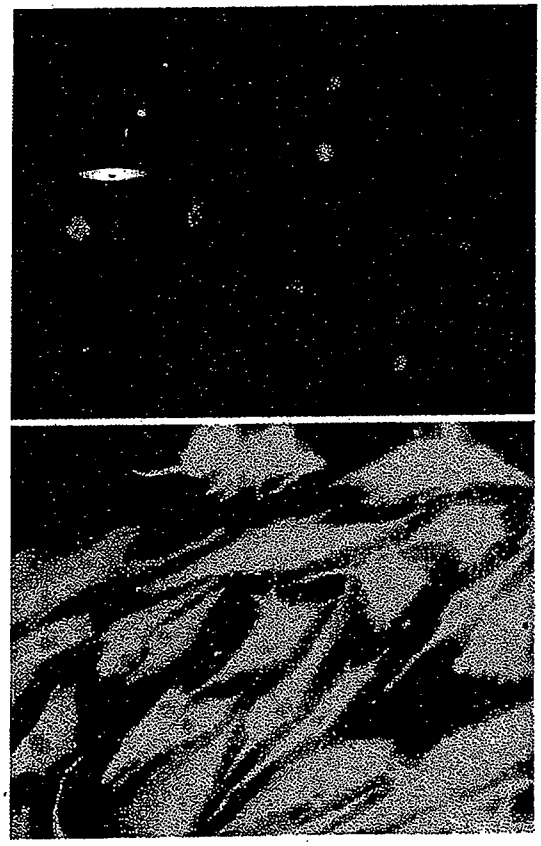

FIG. 2. Immunostaining pattern of RA-NLC with a monoclonal antibody purified from culture supernatant of B-cell clone. Positive reaction was seen using FITC-conjugated anti-human immunoglobulin antibody, using supernatant from (a) Clone RA32a (b) Clone RA79a. Original magnification, ×100.

We purified immunoglobulins from culture supernatants by column chromatography and conducted immunocytochemical staining using antibodies secreted by these B-cell clones to examine their reactivity to human cell lines.

A monoclonal antibody purified from the culture supernatant of the RA32 B-cell clone showed speckled nuclear staining, while eight purified antibodies from the other RA patients showed a diffuse cytoplasmic pattern of RA-NLC (Table 4, Fig. 2).

as supplementary data

TABLE 6. Nucleotide and amino acid sequences of CDR3 region of established B-cell clones

Clone	Sequence of CDR3 region																														
RA3a	GCG	AGA	GGG	TAT	AGT	GCG	ACC	CTC	GGC	GGA	AAC	TTT	GAC	TAC																	
	A	R	G	Y	S	A	T	L	G	G	N	F	D	Y																	
RA32a	GCA	CGA	GGA	TTG	GAT	AGC	AGC	AGC	TGG	AAC	GTT	TTT	GAC	TAC																	
	A	R	G	L	D	S	S	S	W	N	V	F	D	Y																	
RA45a	GCA	AAA	GAC	AGG	AAT	GCT	GGG	ACC	TTC	GAA	AGC	TTA	GAC	TCC																	
	A	K	D	R	N	A	G	T	F	E	S	L	D	S																	
RA79a	GCG	AGG	AGC	GGT	TTC	GGG	GAA	TTA	CCT	CTT	GAC	TAC	GAC																		
	A	R	S	G	F	G	E	L	P	L	D	Y	D																		
RA133a	GCT	CTG	GCC	CAC	TAC	GAC	CTT	GAT	AGT	AGT	GGT	TAT	GAC	TTG	TCG	GGT	CTT	AAT	TAC												
	A	L	A	H	Y	D	L	D	S	S	G	Y	D	L	S	G	L	N	Y												
RA133b	ACA	AGA	TGG	GAG	CAG	CTC	GGT	GGC	TGG	TTC	AAC	CTC																			
	T	R	W	E	Q	L	G	G	W	F	N	L																			
RA133c	GCG	AGA	GTT	TCG	ACC	GGA	GGG	GGA	TTT	TTT	ACT	GAA	GGT	GGT	GCC	TAT	AAT	GCT	TTT	GAT	TTG										
	A	R	V	S	T	G	G	G	F	F	T	E	C	G	A	Y	N	A	F	D	L										
RA133d	GCG	AGA	CCG	GGG	GGG	GCT	TTT	GAT	GTC																						
	A	R	P	F	F	A	F	D	V																						

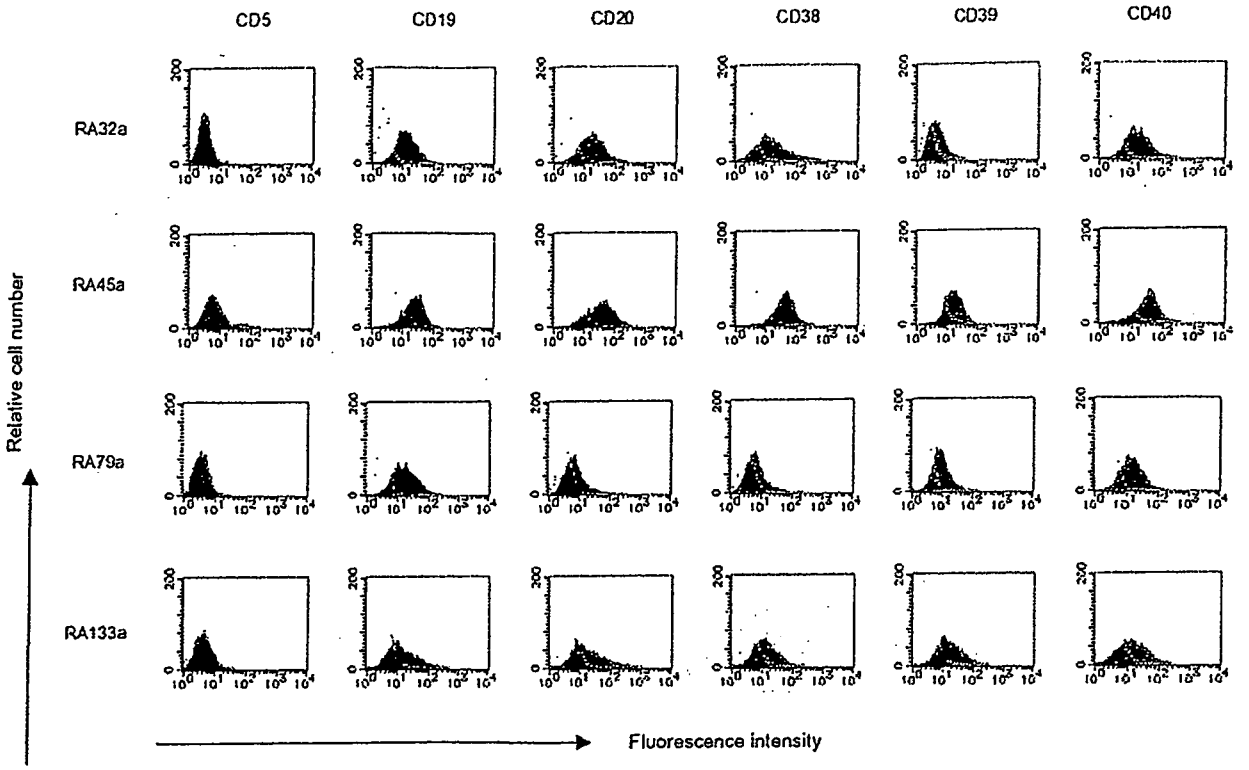

FIG. 3. Phenotypic analysis of B-cell clones for B-cell-specific markers. Horizontal and vertical axes illustrate log fluorescence and relative cell numbers, respectively. The histogram corresponding to each monoclonal antibody (shaded area) is superimposed on that of the negative control (open area) stained with an isotype-matched unrelated monoclonal antibody. Each row represents data from an individual RA patient.

All antibodies from culture supernatants stained human stromal cell lines established from other tissues, i.e. HEp-2, SiHa, Hs729 and ACHN. Our data suggest that the antibodies produced by the B-cell clones recognized antigens that were ubiquitously expressed in various tissues (data not shown).

Surface phenotype of B-cell clones

Individual B-cell clones showed a similar phenotype of cell-surface antigens (Figs 3 and 4). Although fluorescent intensities of antigens were slightly different from clone to clone, all clones expressed B-cell surface markers CD19, CD20 and CD40, which were not expressed on plasma cells. These B-cell clones showed a unique profile, staining double-positive for CD38 and CD39. Antigens CD38 and CD39 are known as markers of germinal centre (GC) B cells, though GC B cells usually expressed either CD38 or CD39 but not both. These clones did not express CD5, a marker of autoreactive B cells in humans [26]. We also analysed a panel of adhesion molecules, including CD11a [lymphocyte function-associated antigen 1 (LFA-1)], CD49d [very late antigen 4 (VLA-4)] and CXCR4, which are believed to be important for B-cell adhesion and pseudoemperipolesis activity involving RA-NLC [17] or fibroblast-like synoviocytes [27]. All clones expressed CD11a and CD49d but not CXCR4, suggesting CXCR4 is not essential for survival of these B-cell clones. In summary, surface marker analysis showed these cloned cells to be activated and mature B cells. Our data also suggest a less important role for the adhesion molecule CXCR4 in the survival of these clones.

EBV transformation of B-cell clones

B-cell clones were examined for EBV transformation by PCR. All samples showed amplification of the *Bam*HI W repeat region of the EBV genome (data not shown).

RA45 antibody reacts with 48-kDa protein expressed in HEp-2 cells

In preliminary experiments, we could not detect any specific reactivity of these antibodies by western blot analysis (data not shown). This suggested that autoantibodies produced by the clones recognized the tertiary structure of the antigen(s). Further examination of the antigen-specificity of autoantibodies produced by B-cell clones was done in immunoprecipitation studies. Using purified RA45 autoantibody and RA-NLC lysate in CTAB buffer, a 48-kDa precipitate was detected (data not shown). This precipitate was also detected in lysate derived from HEp-2 cell, consistent with the antigen specificity observed in immunocytochemical staining. Antibodies purified from culture supernatants of other B-cell clones did not show significant antigen specificity. Identification of the 48-kDa precipitate is now under way.

Discussion

In this study, we reported the establishment of B-cell clones from synovium and bone marrow of patients with RA. Previous studies have looked at the clonal analysis of B cells in the synovium of RA

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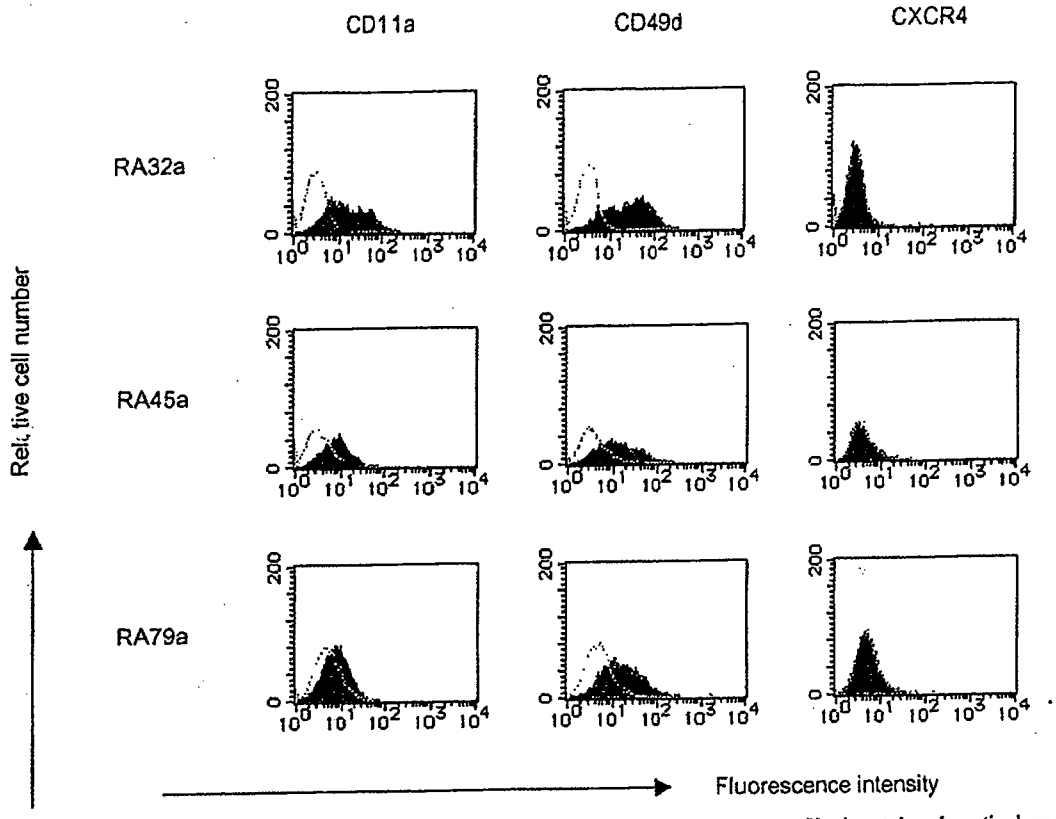

FIG. 4. Phenotypic analysis of B-cell clones for adhesion molecules and/or chemokine receptors. Horizontal and vertical axes illustrate log fluorescence intensity and relative cell numbers, respectively. The histogram corresponding to each monoclonal antibody (shaded area) is superimposed on that of the negative control (open area) stained with an isotype-matched unrelated monoclonal antibody. Each row represents data from one patient.

patients, but this is the first study to do this analysis on B-cell clones. These B-cell lines showed interesting characteristics, such as CD38 and CD39 double-positive phenotypes, autoantibody production, a restricted VH repertoire, and dependence on direct contact with RA-NLC for proliferation, differentiation and activation.

A large diversity of immunoglobulin is generated by recombination between V, D and J segments. However, immunoglobulin repertoire usage is not a random process. The VH3 family is used most frequently, followed by ~~VH~~ (VH4) and VH1 in adult peripheral blood [28, 29]. Also, in each VH family, biased usage of particular VH gene segments was seen; for example, in the VH3 family, the 3-23 gene was most frequently used by healthy adults in peripheral blood [28]. While the 3-23 gene was found most frequently in the peripheral blood [30] and in the synovial tissue of patients with RA [31], we did not find the same germ-line gene in established B-cell lines and clones (Tables 1 and 2). Huang *et al.* [30] showed a lower frequency of the 3-30 gene in RA patients than in healthy donors. However, we detected 3-30 gene usage in two out of nine clones. Our data suggest that the process by which B-cell clones survive is not a random one.

B cells in the human fetal liver are believed to have highly restricted sets of VH gene segments [19–21], and these limited VH segments were widely used for various autoantibodies, including RF, anti-DNA and anti-thyrotropin receptor antibodies [22]. Our established B-cell clones recognized ubiquitous antigens. Consistent with these findings, autoreactive B-cell clones of this study used VH genes which were also frequently found in the fetal liver.

Autoreactive B cells are not always $CD5^+$ [32], though $CD5^+$ B cells have been reported to secrete autoantibodies in several autoimmune diseases [26]. The percentage of $CD5^+$ cells increased in the peripheral blood of RA patients with $CD5^+$ B cells detected in the synovium [33]. Natural antibodies are also produced by $CD5^+$ B cells [34]. In this study, B-cell clones from RA patients did not express CD5 ~~(Figs 3 and 4)~~. (Fig. 3)

Kim and colleagues reported three different subsets of infiltrating B cells in inflamed synovium [31]: (i) terminally differentiated plasma cells ($CD20^-$, $CD38^+$); (ii) mature $CD20^+$, $CD38^-$ B cells; and (iii) activated B cells with GC phenotypes ($CD20^+$ and $CD38^+$ or $CD39^+$). Our B-cell clones expressed CD19, CD20, CD38, CD39 and CD40, which suggests mature, activated cells. Instead of the similarities between RA synovia and lymph nodes, the surface antigens of our B-cell clones differed from those of these activated B cells. This unique expression pattern of surface antigens might be due to EBV, since EBV has been shown to induce B-cell activation [35]. Although our established clones were all transformed by EBV, they rapidly died when cultured in medium alone. Further study of this unique B cell phenotype in the peripheral blood and joints of RA patients is under way.

Rheumatoid synovia can support differentiation of activated B cells into plasma cells [12, 36–38]. RA-NLC and fibroblast-like cells found in RA synovia rescue B cells from apoptosis and have similar phenotypes to follicular dendritic cells in GC [17, 39]. For proliferation, activation and differentiation of these cloned B cells, direct contact and pseudoemperipolesis between B cells and RA-NLC were necessary. In RA-NLC and B-cell interactions, LFA-1-ICAM-1 (intercellular adhesion molecule-1 protein)