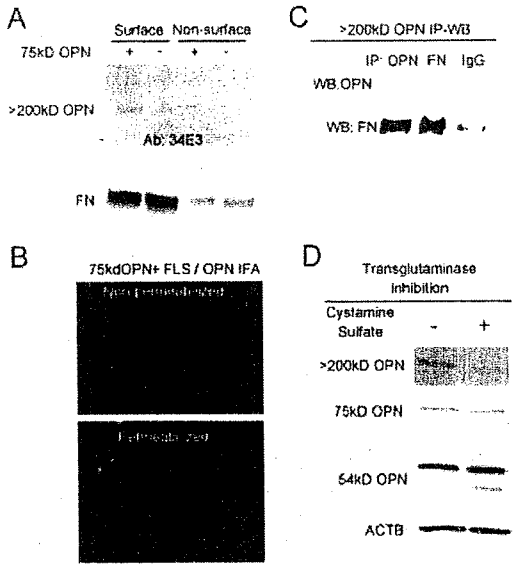

Figure 2. Characteristics of the 75-kd OPN. **A,** Isolation of cell surface proteins was followed by Western blotting with or without the antibody (Ab) directed against the thrombin-cleaved neoepitope of OPN (34E3). The >200-kd form of OPN was detected in the surface protein fraction of 75-kd OPN–positive FLS. Fibronectin (FN) expression was used as the loading control. **B,** Immunofluorescence assay (IFA) was used to assess the expression pattern of OPN on 75-kd OPN–positive FLS stained with OPN antibody O-17, with results showing a distribution of OPN corresponding to the cell outline, regardless of permeabilization. **C,** When >200-kd OPN was further examined by immunoprecipitation–Western blotting (IP-WB), the >200-kd band was detected in the immunoprecipitant of the OPN antibody (O-17) blotted with the FN antibody, and vice versa. Normal rabbit IgG was used as the negative control. **D,** The OPN–positive FLS were treated with or without a transglutaminase inhibitor, cystamine sulfate, and assessed by Western blotting. Expression of >200-kd OPN was reduced by treatment with cystamine sulfate, whereas the expression levels of 75-kd and 54-kd OPN were not altered. See Figure 1 for other definitions.

covalently crosslinked to fibronectin by transglutamination through 2 widely conserved glutamine residues at its N-terminus (26). Therefore, immunoprecipitation of total cell lysates from 75-kd OPN–positive FLS with the anti-OPN or antifibronectin antibody was performed, and this was followed by Western blotting with these antibodies. The >200-kd band was detected when the immunoprecipitant obtained with the antifibronectin antibody was probed by the anti-OPN antibody, and vice versa (Figure 2C). A transglutaminase inhibitor, cystamine sulfate, reduced the expression of >200-kd OPN when the inhibitor was added to the FLS cultures, but did not alter the levels of 75-kd or 54-kd OPN (Figure 2D).

Enhanced IL-6 production by OPN overexpression in 75-kd OPN–positive FLS, but not in 75-kd OPN–negative FLS, in cocultures with B lymphocytes. To elucidate the role of OPN in FLS–B lymphocyte cocultures, gain of function experiments were performed by inducing overexpression of OPN with a lentiviral vector. Western blotting of the cells after lentiviral infection showed that all 75-kd OPN–positive FLS showed up-regulation of the 75-kd, >200-kd, and 54-kd OPN bands, along with an extra band at 50 kd, whereas the 75-kd and >200-kd bands were not up-regulated in any of the 75-kd OPN–negative FLS (Figure 3A).

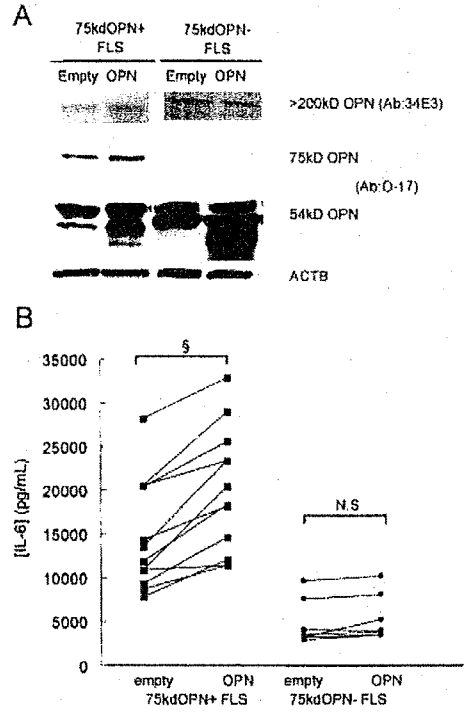

Figure 3. Gain of function experiments, targeting OPN by lentiviral infection with an overexpression vector. **A,** The 75-kd OPN–positive and 75-kd OPN–negative FLS were subjected to lentiviral infection with an empty vector or OPN-overexpressing vector and then assessed by Western blotting. The 54-kd form of OPN, with an extra lower band, showed increased expression in both types of FLS, while >200-kd OPN and 75-kd OPN were increased only in 75-kd OPN–positive FLS. **B,** After lentiviral infection of the FLS and then coculture in contact with B lymphocytes, the IL-6 concentration in the culture supernatant was evaluated. IL-6 levels were significantly increased by OPN overexpression in 75-kd OPN–positive FLS, whereas the levels were not altered in 75-kd OPN–negative FLS. Solid squares show individual FLS samples. § = $P < 0.001$ by 2-factorial analysis of variance. NS = not significant (see Figure 1 for other definitions).

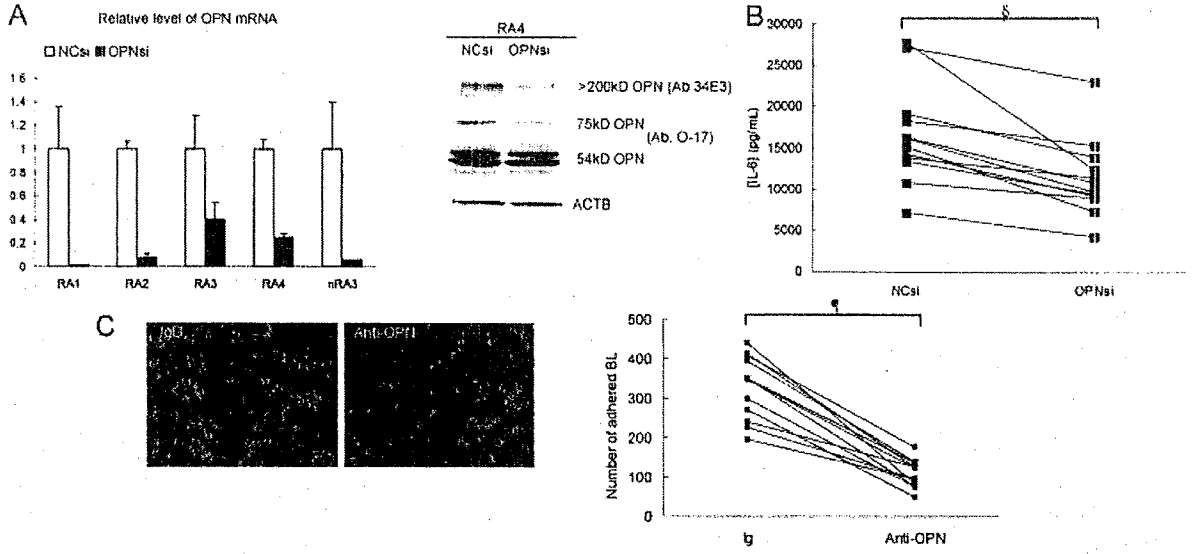

Figure 4. Loss of function experiments, targeting OPN by small interfering RNA (siRNA) (A and B) and a neutralizing antibody (C). A, OPN knockdown in 75-kd OPN–positive FLS by transfection of OPN siRNA (OPNsi) was evaluated by real-time reverse transcription–polymerase chain reaction (left) and by Western blotting (right). Bars show the mean and SD OPN mRNA levels in triplicate experiments with each of 4 RA FLS samples and 1 non-RA (nRA) FLS sample. NCsi = negative control siRNA. B, FLS transfected with negative control siRNA or OPN siRNA were cocultured in contact with B lymphocytes and the IL-6 concentration in the supernatant was evaluated. The IL-6 level was significantly reduced by OPN knockdown. § = $P < 0.001$ by 2-factorial analysis of variance. C, FLS treated with an OPN-neutralizing antibody or class-matched normal IgG were cocultured in contact with B lymphocytes. After removal of nonadherent B lymphocytes by vigorous washing, adherent B lymphocytes were counted in 3 separate fields per well, as viewed under an inverted microscope (left; original magnification × 100). Small round cells are B lymphocytes. The number of adherent B lymphocytes was expressed as the mean (right). Solid squares show individual FLS samples. ¶ = $P < 0.001$ by 2-factorial analysis of variance. See Figure 1 for other definitions.

In addition, all 75-kd OPN–positive FLS showed a significant increase in IL-6 production after OPN overexpression followed by coculture in contact with B lymphocytes, when compared with cells without overexpression ($P < 0.001$ by 2-factorial ANOVA) (Figure 3B). In 75-kd OPN–negative FLS, however, IL-6 production was not altered by overexpression of OPN, which was consistent with the lack of a 75-kd band and the unaltered >200-kd band on Western blotting.

Significant reduction in IL-6 levels following loss of function of OPN in 75-kd OPN–positive FLS, and inhibition of adhesion of B lymphocytes on FLS in cocultures with B lymphocytes. Loss of function experiments were performed using siRNA transfection and an OPN-neutralizing antibody. Transfection of OPN siRNA successfully knocked down OPN expression at the mRNA level as well as at the protein level in all of the 75-kd OPN–positive FLS (Figure 4A). After subsequent coculture of the FLS in contact with B lymphocytes, there was significantly lower IL-6 production by OPN–knocked down FLS compared with that by FLS transfected with nontargeting siRNA ($P < 0.001$ by 2-factorial ANOVA) (Figure 4B).

Adhesion of B lymphocytes to FLS occurred in FLS–B lymphocyte cocultures, as has been reported previously (9,12), but was inhibited by the blocking antibody directed against OPN. The number of B lymphocytes adherent to FLS was significantly reduced by the anti-OPN antibody in all 75-kd OPN–positive FLS ($P < 0.001$ by 2-factorial ANOVA) (Figure 4C).

Detection of IL-6–positive cells in the sublining region of RA synovium, at the site of OPN–B lymphocyte colocalization. Finally, to assess the distribution of OPN, IL-6, and B lymphocytes in the RA synovium, immunohistochemical analyses were carried out using anti–IL-6, anti-OPN, and anti–pan B lymphocyte antigen CD79α antibodies. One of the synovial villi located in the knee joint from an RA patient was studied. As reported previously, OPN was distributed in the fibroblastic cells and the matrix of the synovial lining and sublining regions (34,35). Clusters of B lymphocytes were found in the deeper layers of the synovium as well as in the

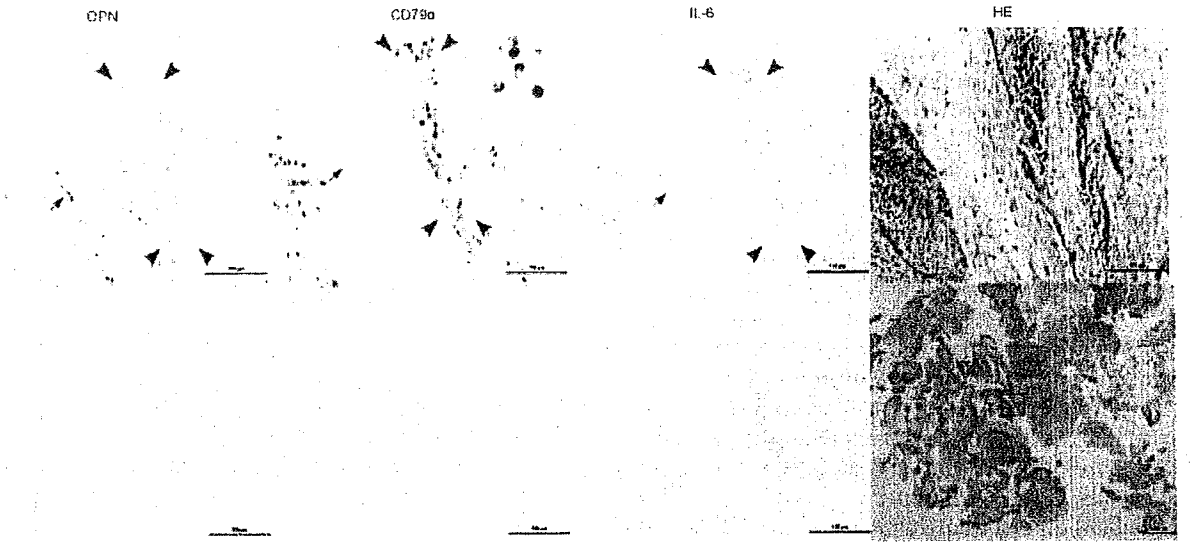

Figure 5. Immunohistochemical analysis of rheumatoid arthritis (RA) synovium. One of the synovial villi in the inflamed joint of a patient with RA was evaluated by hematoxylin and eosin (H&E) staining. The H&E-stained image in the upper right panel is a higher-magnification view of the boxed area in the lower right panel. The RA synovium samples in the other 3 upper panels were stained with the antibody against osteopontin (anti-OPN) (O-17), anti-pan B cell antigen CD79α, and anti-interleukin-6 (anti–IL-6) antibodies. The corresponding panels below were stained with class-matched normal IgG as a negative control. The appearance of IL-6–positive cells coincided with the site at which OPN and B lymphocytes colocalized (**arrowheads**), whereas IL-6 was not detected in areas where only OPN-positive cells were distributed or at sites far from clusters of B lymphocytes (**arrows**).

sublining region, and some of these B lymphocyte clusters were surrounded by OPN-positive cells. IL-6–positive cells were scattered in the sublining region, as has been reported previously (44).

Of note, some of the areas of distribution of IL-6–positive cells coincided with sites at which OPN and B lymphocytes colocalized (indicated by arrowheads in Figure 5). However, IL-6 was not detected in areas where only OPN-positive cells were distributed or at sites far from clusters of B lymphocytes (indicated by arrows in Figure 5).

DISCUSSION

The present study revealed a new role of OPN in the RA synovium, using primary cultured FLS derived from RA patients to analyze the function of FLS-expressed native OPN. A specifically modified 75-kd OPN was predominantly expressed by RA FLS and was associated with significantly elevated IL-6 production in FLS–B lymphocyte cocultures. The 75-kd OPN formed a >200-kd OPN/fibronectin–crosslinked molecule via transglutamination, which was detected on the synovial cell surface, resulting in exposure of its thrombin-cleaved neoepitope. This >200-kd cell surface OPN affected the interactions between FLS and B lymphocytes by supporting adhesion of B lymphocytes to FLS in FLS–B lymphocyte cocultures, and consequently IL-6 production was enhanced. This FLS–B lymphocyte interaction also appeared to occur in vivo.

OPN by itself weighs 37 kd, as determined previously using mass spectrometry (25), but due to its various posttranslational modifications, the migration of full-length OPN, detected on SDS-PAGE, differs among cell types within ~45–80 kd (42). Accordingly, our results from Western blotting of OPN showed multiple bands with different molecular weights. In particular, a single band at 75 kd that was detected predominantly in RA FLS suggested that this specifically modified 75-kd OPN was worth investigating to determine the function of OPN in RA.

In fact, the localization of OPN was different between 75-kd OPN–negative and 75-kd OPN–positive FLS. The existence of >200-kd OPN in the cell surface fraction of 75-kd OPN–positive FLS on Western blotting, and the staining pattern of OPN on immunofluorescence assay of these FLS, showing the cell outline distribution, suggested that OPN was associated with the cell surface side of the plasma membrane. These distinct

findings were considered attributable to 75-kd OPN, since they were negative in 75-kd OPN–negative FLS. Such posttranslational modification–dependent differences in subcellular localization of OPN have also been observed in previously published studies of OPN expressed by normal rat kidney (NRK) cells, which revealed that among phosphorylated and nonphosphorylated forms of OPN expressed by NRK cells, only phosphorylated OPN was associated with the cell surface (19,23). Moreover, another study revealed that OPN was detected at >200 kd molecular weight and found to be localized on the surface of NRK cells when covalently crosslinked to fibronectin by a transglutaminase (26). Our results were consistent with that study, since the observations from immunoprecipitation and Western blotting of the FLS suggested that >200-kd OPN was an OPN/fibronectin-crosslinked molecule, and inhibition of a transglutaminase reduced the expression of >200-kd OPN. Considering these facts together, >200-kd OPN could be considered an OPN/fibronectin–covalently crosslinked molecule synthesized by a transglutaminase from 75-kd OPN.

In contrast, >200-kd cell surface OPN was poorly detected in 75-kd OPN–negative FLS. Moreover, overexpression of OPN by 75-kd OPN–negative FLS only increased the expression of 54-kd OPN, and did not induce expression of 75-kd OPN nor did it alter the expression of >200-kd OPN, suggesting that there might be an unknown enzyme in 75-kd OPN–positive FLS that performs the specific modifications of 75-kd OPN necessary for it to associate with the cell surface and to form the >200-kd OPN/fibronectin–crosslinked molecule.

To assess the relevance of 75-kd OPN in RA, we performed cocultures of FLS and B lymphocytes. Previous studies have revealed that FLS–B lymphocyte coculture allows the adhesion of B lymphocytes to FLS, which induces cell–cell interactions between FLS and B lymphocytes and, consequently, increases the production of several cytokines, including IL-6 (9,12), but the mechanism has not been elucidated. We focused on IL-6 production in FLS–B lymphocyte cocultures and found that increased IL-6 production was associated with the existence of 75-kd OPN, which suggested that 75-kd OPN together with cell–cell interactions between FLS and B lymphocytes can enhance IL-6 production. We also revealed that among FLS and B lymphocytes, FLS were the dominant cell type for IL-6 production in coculture, as was shown in experiments involving knockdown of IL-6 in FLS.

To examine whether 75-kd OPN could enhance IL-6 production, we performed overexpression and knockdown of OPN in FLS, which demonstrated that IL-6 production was increased and decreased in accordance with the increase and decrease of 75-kd OPN and >200-kd OPN, respectively. Among these 2 OPN forms with different molecular weights, >200-kd OPN appeared to enhance IL-6 production, since the transglutaminase inhibitor that reduced the expression of only the >200-kd OPN significantly suppressed IL-6 production in 75-kd OPN–positive FLS–B lymphocyte cocultures (results not shown).

We then analyzed how 75-kd OPN or its crosslinked form, >200-kd OPN, enhanced IL-6 production in FLS–B lymphocyte cocultures. The >200-kd OPN on the cell surface exposed its thrombin-cleaved neoepitope, SVVYGLR (27), a ligand for integrin $\alpha 4\beta 1$, also known as VLA-4, integrin $\alpha 9\beta 1$, and integrin $\alpha 4\beta 7$ (30). Considering the fact that VLA-4 is expressed by B lymphocytes and supports adhesion of B lymphocytes to FLS in FLS–B lymphocyte coculture (12), >200-kd OPN was indicated as the ligand for VLA-4, which acts as an adhesion molecule. This idea was supported by the results from our B lymphocyte adhesion assay with a blocking antibody against OPN, and by the fact that the transglutaminase inhibitor also suppressed adhesion of B lymphocytes to FLS (results not shown). Meanwhile,

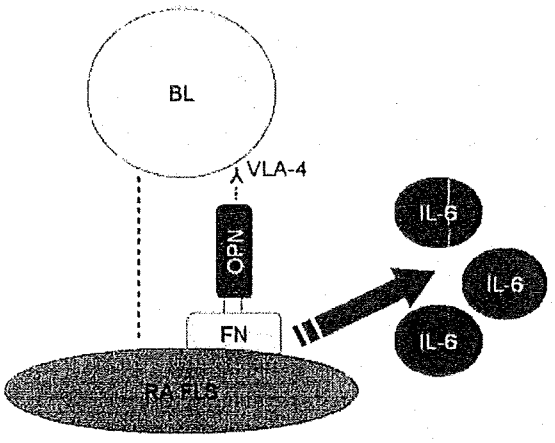

Figure 6. Diagram of the in vitro findings, showing cell–cell interactions between fibroblast-like synoviocytes (FLS) and B lymphocytes (BL). RA FLS were characterized by the expression of 75-kd OPN, from which a transglutaminase synthesized the >200-kd OPN/fibronectin (FN)–crosslinked molecule that was localized on the synovial cell surface in its thrombin-cleaved form. This cell surface OPN mediated cell–cell interactions between OPN and B lymphocytes by supporting adhesion of B lymphocytes to FLS through very late activation antigen 4 (VLA-4) and enhanced IL-6 production. An unknown link between FLS and B lymphocytes is also shown. See Figure 5 for other definitions.

integrin $\alpha4\beta7$, which was also expressed by B lymphocytes, did not mediate such adhesion (12).

VCAM-1, which was expressed by FLS and also supports B lymphocyte adhesion, possibly through VLA-4 (11), was detected in equal amounts among RA and non-RA FLS (results not shown). Therefore, we postulated that integrin $\alpha4\beta7$ and VCAM-1 were not involved in the adhesion of B lymphocytes to FLS or subsequent IL-6 production, and that such adhesion was mediated by >200-kd OPN and VLA-4, which would further initiate the cell–cell interaction between FLS and B lymphocytes, leading to IL-6 production.

Our findings suggested that, in response to these FLS–B lymphocyte interactions, FLS boosted their IL-6 production. Similar studies on cell–cell interactions between FLS and T lymphocytes have shown that lymphocyte function–associated antigen 1, intercellular adhesion molecule 2, and the ezrin/Akt pathway are involved in their interaction, which also enhances IL-6 production (2). Thus, this pathway may also be involved in FLS–B lymphocyte interactions, although further investigation is needed to fully elucidate the mechanism.

In summary, the findings from these in vitro experiments showed that RA FLS are characterized by the expression of 75-kd OPN. This was the substrate of a transglutaminase that formed the >200-kd OPN/fibronectin–crosslinked molecule, a molecule localized on the surface of FLS in its thrombin-cleaved form. This surface OPN mediated cell–cell interactions between FLS and B lymphocytes, and enhanced IL-6 production (Figure 6). Moreover, such FLS–B lymphocyte interactions, or interactions between FLS-expressing OPN and B lymphocytes stimulating IL-6 production, appeared to take place in vivo, as shown by immunohistochemistry. Taking into account the previously reported findings on the pathogenic significance of IL-6 (45), B lymphocytes (46,47), VLA-4 (48), and the thrombin-cleaved neoepitope of OPN (32,33) in RA, the present study revealed a novel role of OPN in RA.

Regarding the weaknesses of this study, we need to mention the discrepancy in the findings between RA FLS and 75-kd OPN–positive FLS. Since 75-kd OPN was detected in all 10 RA FLS, but also in 3 non-RA FLS, it was not specific to RA FLS. There is no doubt that 75-kd OPN enhanced IL-6 production in FLS–B lymphocyte cocultures and appeared to aggravate chronic inflammation in vivo. However, it should not be considered the cause of RA; rather, we could postulate that it is one of several molecules with induced expression in arthritis and is involved in the chronic progression of arthritis by stimulating IL-6 production. This is supported by the observation that, in the joint tissue of a non-RA donor, the FLS expressed amounts of 75-kd OPN comparable with those in RA FLS, and showed severe synovitis at the time of surgery.

We also have to note that the mechanism involved in the modification of 75-kd OPN has not been defined, and the responsible transglutaminase has not been identified. Further investigations in this area would be required for better understanding of the pathology of RA. Nevertheless, our results show that a specifically modified 75-kd form of OPN was expressed by RA FLS. This form of OPN affected cell–cell interactions between FLS and B lymphocytes by supporting the adhesion of B lymphocytes to FLS. As a result, IL-6 production was enhanced in FLS–B lymphocyte cocultures.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Nakata had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Nakata, Ochi, Yoshikawa.
Acquisition of data. Take, Nakata, Nishimoto.
Analysis and interpretation of data. Take, Nakata, Hashimoto, Tsuboi, Nishimoto.

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