An Essential Role for STAT6-STAT1 Protein Signaling in Promoting Macrophage Cell-Cell Fusion

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Macrophage lineage cells such as osteoclasts and foreign body giant cells (FBGCs) form multinuclear cells by cell-cell fusion of mononuclear cells. Recently, we reported that two seven-transmembrane molecule, osteoclast stimulatory transmembrane protein (OC-STAMP), were identified as required for cell-cell fusion of both mononuclear cells and osteoclasts by generating knock-out mice (1). The function of FBGC cell-cell fusion is likely removal or degradation of foreign materials (2, 3). FBGCs form in response to Th2 cytokines such as IL-4 and IL-13 (4–6), and signal transducer and activator of transcription factor (STAT)-6 plays an essential role in transducing IL-4/IL-4R signals. However, mechanisms underlying FBGC cell-cell fusion remain largely unknown.

To date, various molecules have been identified as regulating macrophage and/or osteoclast cell-cell fusion. Macrophage fusion receptor (MFR; also called SHIPS-1), a member of immunoglobulin (Ig) superfamily, was shown to function in macrophage cell-cell fusion based on loss-of-function assays using MFR monoclonal antibodies or the soluble form of the extracellular domain of MFR (7, 8). CD47, an MFR ligand, and the intracellular domain of CD44 (CD44ICD) have also been implicated in macrophage cell-cell fusion (9, 10). DAP12, an ITAM motif-containing adaptor protein, and the signaling molecule Syk were also reportedly involved in cell-cell fusion of macrophages (11). Furthermore, studies employing antisense oligonucleotides and knock-out mice showed that Meltrin α (also called ADAM12) and matrix metalloprotease 9 (MMP9) were involved in multinucleation of macrophages and osteoclasts, respectively (12, 13). Defective fusion of macrophages and osteoclasts was also demonstrated in MCP-1/CCL2 or ATP6v0d2 knock-out cells (14, 15). In studies of osteoclasts employing neutralizing antibody and knock-out mice, respectively, E-cadherin and CD200 were reported to play a role in cell-cell fusion (16, 17). Recently, two multimembrane-spanning molecules, dendritic cell-specific transmembrane protein (DC-STAMP) and osteoclast stimulatory transmembrane protein (OC-STAMP), were identified as required for cell-cell fusion of both macrophages and osteoclasts by generating knock-out mice (18, 19).

Here, we employ STAT1-deficient and STAT6-deficient mice to show that STAT6 activation by IL-4 inhibits STAT1,
leading to expression of OC-STAMP and DC-STAMP in macrophages. STAT1 deficiency or co-expression of OC-STAMP and DC-STAMP was sufficient to promote macrophage cell-cell fusion without IL-4. These observations define molecular mechanisms underlying cell-cell fusion of macrophages.

EXPERIMENTAL PROCEDURES

Mice—STAT1-deficient and STAT6-deficient mice were purchased from Taconic (Hudson, NY) and The Jackson Laboratory (Bar Harbor, ME), respectively. OC-STAMP Tg mice were generated as described previously (19). Animals were maintained under specific pathogen-free conditions in animal facilities certified by the Keio University School of Medicine Animal Care Committee. Animal protocols were approved by that committee.

Reagents—M-CSF, RANKL, GM-CSF, and IL-4 were purchased from R&D Systems Inc. (Minneapolis, MN). FK506 was purchased from Astellas Pharma (Tokyo, Japan).

In Vitro Osteoclastogenesis and Foreign Body Giant Cell Formation—Cell culture and tartrate resistance acid phosphatase and May-Grünwald Giemsa staining were performed as described previously (18, 20–23). Briefly, BM cells were cultured in the presence of M-CSF (50 ng/ml) for 3 days and then harvested as common progenitors of osteoclasts and FBGCs. Progenitors were cultured in the presence of M-CSF alone (50 ng/ml) for macrophages, M-CSF (50 ng/ml) + RANKL (25 ng/ml) for osteoclasts, GM-CSF alone (50 ng/ml) for immature dendritic cells, and GM-CSF (50 ng/ml) + IL-4 (50 ng/ml) for FBGCs.

FBGC Formation in Vivo—A PVA sponge was implanted subcutaneously in WT, STAT6-deficient, and STAT1-deficient mice. After 3 days, implants were harvested, and histological analysis was performed using hematoxylin and eosin staining.

Real Time PCR and RT-PCR Analysis—β-Actin and GAPDH expression served as internal controls for real time PCR and RT-PCR, respectively. Primer sequences were as follows for real time PCR: β-actin forward, 5'-TGAAGGGGAATATTGCGTGAC-3', and β-actin reverse, 5'-AAGAGGAGGCTGGAAAGAG-3'; OC-STAMP forward, 5'-ATGAGGACCATCACGGGCAGCCACG, and OC-STAMP reverse, 5'-GGAGAGAGGTTCATTCCAA-3'; and GAPDH reverse, 5'-ACTAGAGGAGAAGTCCTGGGAGTC-3'. Primer sequences for RT-PCR were as follows: Actin and GAPDH primers as described above, 5'-TGAGGAGGGAAATCGTGCG-3', and 5'-TGAGGAGGGAAATCGTGCG-3', respectively.

RESULTS

OC-STAMP Expression in FBGCs Requires STAT6—DC-STAMP and OC-STAMP are required for cell-cell fusion of macrophage lineage cells (18, 19), and first we analyzed the expression of these molecules in bone marrow macrophages (BMMs) treated with M-CSF, GM-CSF, or GM-CSF plus IL-4 in vitro (Fig. 1). DC-STAMP is reportedly expressed in GM-CSF-induced dendritic cells (24) but not in M-CSF-induced mononuclear macrophages (18). Indeed, DC-STAMP expression was significantly higher in GM-CSF-treated compared with M-CSF-treated BMMs and was further up-regulated by IL-4 in addition to GM-CSF, a condition known to induce FBGCs (Fig. 1A). By contrast, although GM-CSF alone did not induce OC-STAMP expression in BMMs, OC-STAMP expression was significantly up-regulated by treatment with GM-CSF plus IL-4 (Fig. 1A). IL-4 signaling is transduced via STAT6 (25–27), suggesting that OC-STAMP expression requires STAT6. We did not detect STAT6 phosphorylation in M-CSF- or GM-CSF-treated BMMs, but we observed strong STAT6 phosphorylation in GM-CSF plus IL-4-treated FBGCs (Fig. 1B). Thus, we analyzed FBGC formation in STAT6-deficient mice and found that multinuclear FBGC formation was abrogated in vitro and in vivo (Fig. 1, C and D). DC-STAMP and OC-STAMP expression, as analyzed by RT-PCR and real time PCR, were both significantly inhibited in STAT6-deficient FBGCs (Fig. 1, E and F), strongly suggesting that both are STAT6 targets. However, although DC-STAMP expression was significantly inhibited in STAT6-deficient FBGCs, residual DC-STAMP expression remained (Fig. 1, E and F), suggesting that DC-STAMP expression is regulated in part by a STAT6-independent mechanism. MMP9, a marker of differentiated FBGCs (13, 28), was expressed in STAT6-deficient cells at levels equivalent to those seen in wild-type cells (supplemental Fig. SI A), suggesting that DC-STAMP and OC-STAMP expression rather than differentiation was specifically inhibited in STAT6-deficient cells. Indeed, STAT6-deficient macrophages exhibited phagocytic activity equivalent to that seen in wild-type cells (supplemental Fig. SI B).

OC-STAMP Expression Is Regulated by Different Mechanisms in FBGCs and Osteoclasts—In contrast to FBGCs, STAT6-deficient osteoclasts showed moderate stimulation rather than inhibition of multinucleation (Fig. 2A). OC-STAMP expression was equivalent in both STAT6-deficient and wild-type osteoclasts (Fig. 2B), suggesting that its expression in osteoclasts is STAT6-independent. Indeed, we found that STAT6 was not activated in osteoclasts (Fig. 2C). In addition, we recently found that OC-STAMP expression in osteoclasts is regulated directly by NFATc1, an transcription factor essential for osteoclastogenesis, and that osteoclast multinucleation and
PVA sponges were implanted subcutaneously in WT and STAT6 KO mice. Three days after implantation, sponges were stained with hematoxylin and eosin (H&E), and the number of multinuclear osteoclasts containing more than three nuclei was scored (right panel). Arrows and arrowheads indicate fused cells and aggregated cells, respectively, **, p < 0.001.

BMMs isolated from wild-type mice were cultured in the presence of GM-CSF (50 ng/ml) or GM-CSF plus IL-4 (GI) for 6 days, and STAT6 phosphorylation was analyzed by Western blot. pSTAT6 expression is differently regulated in osteoclasts and FBGCs.

DC-STAMP and OC-STAMP expression in FBGCs derived from wild-type (+/+) or STAT6-KO (−/−) mice was analyzed by real time PCR. **, p < 0.001.

STAT1 Negatively Regulates Cell-Cell Fusion and DC-STAMP/OC-STAMP Expression in FBGCs—Next, we analyzed the role of STAT1 in FBGC fusion, because STAT1 is reportedly activated in dendritic cells induced following stimulation with GM-CSF plus IL-4 (29). STAT1 phosphorylation levels in cells treated with GM-CSF plus IL-4 were similar to that seen in cells treated with GM-CSF alone and was higher than in cells treated with M-CSF alone (Fig. 4A). Because STAT1 was phos-
Function of STAT6-STAT1 Axis in Macrophage Fusion

STAT1 deficiency is sufficient to promote macrophage cell-cell fusion without IL-4. A–C, BMMs were isolated from wild-type (WT) and STAT1-deficient (STAT1 KO) mice and cultured in the presence of GM-CSF alone for 7 days. Cells were then stained with May-Grunwald Giemsa (A, left panels), and the number of multinuclear FBGCs containing more than three nuclei was scored (A, right panel). **, p < 0.001. Bar, 50 μm. D, DC-STAMP and OC-STAMP expression was analyzed by real time PCR. NC, no template control (B) and by real time PCR. **, p < 0.001 (C). D, BMMs were isolated from wild-type (WT) and STAT1-deficient (KO) mice, cultured in the presence of GM-CSF alone or GM-CSF + IL-4 for 7 days, and analyzed for STAT6 phosphorylation by Western blot. E, BMMs were isolated from wild-type (WT) and STAT6-deficient (KO) mice, cultured in the presence of GM-CSF + IL-4 for 7 days, and analyzed for STAT1 phosphorylation by Western blot.

DC-STAMP and OC-STAMP Co-expression Is Sufficient for Cell-Cell Fusion of Macrophages—Finally, we asked whether combined expression of OC-STAMP and DC-STAMP was sufficient for macrophage fusion in the absence of IL-4, because fusion correlates with increased OC-STAMP and DC-STAMP expression in STAT1-deficient macrophages in the presence of GM-CSF alone. BMMs isolated from wild-type or OC-STAMP Tg mice were cultured in the presence of GM-CSF alone (Fig. 6A). In OC-STAMP Tg cells, OC-STAMP expression was driven by the actin promoter, whereas endogenous DC-STAMP expression was induced by GM-CSF in both OC-STAMP Tg and wild-type cells (Fig. 6A). In these conditions,
MMP9 expression was detected in both DC-STAMP Tg and wild-type cells (supplemental Fig. S5); however, cell-cell fusion occurred only in OC-STAMP Tg but not in wild-type cells (Fig. 6A). Although FBGC cell-cell fusion was completely abrogated in DC-STAMP-deficient and OC-STAMP-deficient cells (supplemental Fig. S6A), STAT6 phosphorylation was detected in DC-STAMP-deficient and OC-STAMP-deficient cells as wild-type FBGCs (supplemental Fig. S6B). Thus, OC-STAMP and DC-STAMP likely cooperate in promoting FBGC fusion through the STAT6-STAT1 axis (Fig. 6B).

DISCUSSION

Multinuclear giant cell formation occurs at sites of implantation or granulomatous inflammation and is a consequence of cell-cell fusion of mononuclear cells (1, 18, 22, 30). However, the mechanisms underlying such fusion have remained largely unknown. We previously identified two transmembrane proteins, OC-STAMP and DC-STAMP, which are essential for osteoclast and macrophage cell-cell fusion, and we showed that lack of either completely abrogated cell-cell fusion of both osteoclasts and FBGCs (18, 19). Here, we show that following IL-4 stimulation, the STAT6-STAT1 axis regulates OC-STAMP and DC-STAMP expression in FBGCs. STAT6 deficiency completely inhibited cell-cell fusion in FBGCs in vivo and in vitro. Lack of STAT6 increased STAT1 activation, which in turn significantly inhibited both OC-STAMP and DC-STAMP expression in FBGCs. Furthermore, STAT1 deficiency was sufficient to promote cell-cell fusion and OC-STAMP and DC-STAMP expression in FBGCs without IL-4, indicating that IL-4 signaling through STAT6 is likely required to inhibit STAT1 and promote fusion by inducing OC-STAMP and DC-STAMP. Indeed, OC-STAMP and DC-STAMP co-expression was sufficient to promote cell-cell fusion in macrophages not treated with IL-4. We conclude that OC-STAMP and DC-STAMP expression and the STAT6-STAT1 signaling cascade are essential for FBGC cell-cell fusion.

STAT1 and STAT6 reportedly reciprocally regulate each other in T cells (31). However, we found that in macrophages, STAT6 regulates STAT1 activation, whereas STAT6 activation is STAT1-independent, suggesting that regulation of signal transducer and activator of transcription factor protein activity differs depending on cell type.

Osteoclasts and FBGCs share common progenitors, and both express the factors such as MMP9, DC-STAMP, OC-STAMP, and ATP6v0d2 (13, 15, 18, 19). In addition, cell-cell fusion in both cells requires DC-STAMP and OC-STAMP (18, 19). However, their respective differentiation is induced by unique cytokine combinations as follows: M-CSF + RANKL for osteoclasts and GM-CSF + IL-4 for FBGCs. Both GM-CSF and IL-4 strongly inhibit osteoclastogenesis, suggesting that different signals govern fusion of osteoclasts and FBGCs. Osteoclast differentiation has been extensively characterized, and c-Fos, a component of AP1, and NFATc1 have been shown to be essential for osteoclastogenesis (32). Stimulation by RANKL, a cytokine essential for osteoclast differentiation, induces and activates NFATc1. We have shown that NFATc1 inhibition by FK506, a calcineurin inhibitor, inhibits cell-cell fusion as well as DC-STAMP and OC-STAMP expression in osteoclasts but not in FBGCs (Fig. 3) (19), suggesting that mechanisms underlying cell-cell fusion mediated by DC-STAMP and OC-STAMP differ in osteoclasts and FBGCs. Indeed, lack of STAT6 completely blocked fusion of FBGCs but not of osteoclasts. Overall, these observations suggest that DC-STAMP and OC-STAMP are required for fusion of osteoclasts and FBGCs via unique signal transducer and activator of transcription factor signaling.

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