Enhanced osteoclastogenesis in patients with MSMD due to impaired response to IFN-γ

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Background: Patients with Mendelian susceptibility to mycobacterial disease (MSMD) experience recurrent and/or persistent infectious diseases associated with poorly virulent mycobacteria. Multifocal osteomyelitis is among the representative manifestations of MSMD. The frequency of multifocal osteomyelitis is especially high in patients with MSMD etiologies that impair cellular response to IFN-γ, such as IFN-γR1, IFN-γR2, or STAT1 deficiency.

Objectives: This study sought to characterize the mechanism underlying multifocal osteomyelitis in MSMD.

Methods: GM colonies prepared from bone marrow mononuclear cells from patients with autosomal dominant (AD) IFN-γR1 deficiency, AD STAT1 deficiency, or STAT1 gain of function (GOF) and from healthy controls were differentiated into osteoclasts in the presence or absence of IFN-γ. The inhibitory effect of IFN-γ on osteoclastogenesis was investigated by quantitative PCR, immunoblotting, tartrate-resistant acid phosphatase staining, and pit formation assays.

Results: Increased osteoclast numbers were identified by examining the histopathology of osteomyelitis in patients with AD IFN-γR1 deficiency or AD STAT1 deficiency. In the presence of receptor activator of nuclear factor kappa-B ligand and M-CSF, GM colonies from patients with AD IFN-γR1 deficiency, AD STAT1 deficiency, or STAT1 GOF differentiated into osteoclasts, similar to GM colonies from healthy volunteers. IFN-γ concentration-dependent inhibition of osteoclast formation was impaired in GM colonies from patients with AD IFN-γR1 deficiency or AD STAT1 deficiency, whereas it was enhanced in GM colonies from patients with STAT1 GOF.

Conclusions: Osteoclast differentiation is increased in AD IFN-γR1 deficiency and AD STAT1 deficiency due to an impaired response to IFN-γ, leading to excessive osteoclast proliferation and, by inference, increased bone resorption in infected foci, which may underlie multifocal osteomyelitis. (J Allergy Clin Immunol 2021;■■■:■■■-■■■.)

Key words: *Mendelian susceptibility to mycobacterial diseases, MSMD, STAT1, IFN-γR1, mycobacteria, osteomyelitis, osteoclastogenesis*

Mendelian susceptibility to mycobacterial disease (MSMD) (Online Mendelian Inheritance in Man no. 209950) is a primary immunodeficiency characterized by susceptibility to clinical disease caused by intramacrophagic pathogens, such as BCG, nontuberculous mycobacteria, or salmonella.[1] To date, 11 MSMD-causing genes (*IFNGR1, IFNGR2, STAT1, IL12B, IL12RB1, ISG15, IRF8, TYK2, SPPL2A, CYBB,* and *IKBKG*) that are involved in IL-12/IFN-γ immune responses have been reported.[2-18] Patients with MSMD experience recurrent and/or persistent infectious diseases associated with poorly virulent mycobacteria, such as BCG and nontuberculous mycobacteria. Indeed, BCG disease after vaccination is frequently found in patients with MSMD.[2] Multifocal osteomyelitis, sometimes with confirmation of the presence of mycobacteria in biopsy specimens, is among the representative and specific manifestations of MSMD.[2,19] An x-ray examination of osteomyelitic regions shows osteolytic changes occasionally surrounded by sclerotic lesions, indicating the presence of chronic osteomyelitis.[20,21] Histopathological analysis of biopsy specimens generally shows granuloma. Although the frequency is relatively low, the presence of occasional acid-fast bacilli has been identified in granulomatous lesions in typical cases.[20] Interestingly, the frequency of multifocal osteomyelitis is especially high in patients with MSMD due to an impaired response to IFN-γ, such as that resulting from IFN-γR1, IFN-γR2, or STAT1 deficiency.[2,19,22,23] Among these disorders, the frequency of bone involvement is high in patients with autosomal dominant (AD) IFN-γR1 deficiency or AD STAT1 deficiency, whereas it is somehow relatively low in patients with complete defect of IFN-γ signaling due to autosomal recessive (AR) IFN-γR1 complete deficiency or AR complete STAT1 deficiency (Table I, and see Tables E1-E3 in this article's Online Repository at www.jacionline.org).[19,22] In addition, it is known that patients with AD IFN-γR1 deficiency typically present with multifocal osteomyelitis predominantly affecting the axial skeleton.[19] On the other hand, salmonellosis is relatively frequent in patients with a deficiency in IL-12Rβ1 or IL-12p40, which are involved in both IL-12 and IL-23

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Abbreviations used
AD: Autosomal dominant
AR: Autosomal recessive
BM-MNCs: Bone marrow derived mononuclear cells
CNO: Chronic nonbacterial osteomyelitis
GOF: Gain of function
IRF: Interferon regulatory factor
MSMD: Mendelian susceptibility to mycobacterial diseases
NFATc1: Nuclear factor of activated T cells, cytoplasmic 1
RANKL: Receptor activator of nuclear factor kappa-B ligand
TRAP: Tartrate-resistant acid phosphatase

signaling.[2,24,25] However, the frequency of bone involvement is low in patients with these 2 disorders.

Among other functions, IFN-γ is a cytokine that can inhibit osteoclastogenesis and osteoclast bone resorption activity in humans and mice.[26-29] In this study, we examined the inhibitory effect of IFN-γ on receptor activator of nuclear factor kappa-B ligand (RANKL)- and M-CSF–mediated osteoclast formation with bone marrow–derived osteoclast precursor cells from patients with AD IFN-γR1 deficiency or AD STAT1 deficiency. Based on the results of current study, we propose a possible link between multifocal osteomyelitis found in patients with MSMD and enhanced osteoclast differentiation due to the loss of suppression triggered by IFN-γ signaling.

METHODS

Patients

One patient with AD IFN-γR1 deficiency (patient 1: heterozygous c.774delTCTA mutation in *IFNGR1*), 2 patients with AD STAT1 deficiency (patient 2: heterozygous p.G250E mutation in *STAT1*, patient 3: heterozygous p.Y701C mutation in *STAT1*), 1 patient with STAT1 gain of function (GOF) (patient 4: heterozygous GOF mutation, p.R274Q, in *STAT1*), and healthy volunteers were enrolled in this study. All 3 patients with AD IFN-γR1 or AD STAT1 deficiency included in this study had clinical episodes of multifocal osteomyelitis. Detailed clinical records are available in previous reports.[22,30-33] Briefly, patient 1 had a history of BCG lymphadenitis at the age of 6 months. She developed multifocal osteomyelitis associated with *Mycobacterium avium* infection at the age of 12 years.[30] Patient 2 developed multifocal osteomyelitis at the age of 2 years. BCG was suspected as a pathogen based on detection of the *M tuberculosis* complex by PCR, with positive tuberculin skin test and negative QuantiFERON-TB2G (Qiagen, Hilden, Germany) test results.[32] Patient 3 developed multifocal osteomyelitis at the age of 3 years.[22] Although the pathogenic bacteria were not isolated, mycobacterial infection was suspected because the clinical symptoms improved in response to antimycobacterial drugs.

Immunohistochemical staining of bone marrow biopsy sections

Immunohistochemical staining for tartrate-resistant acid phosphatase (TRAP) using 3,3'-diaminobenzidine was performed on ethanol-fixed frozen bone marrow biopsy sections. The sections were fixed with 95% ethanol at room temperature for 10 minutes. To block activity of endogenous enzymes, the sections were treated with 3% H_2O_2 at room temperature for 5 minutes. The sections were then treated with Blocking One (Nacalai Tesque, Kyoto, Japan) and incubated with anti-TRAP mouse monoclonal antibodies (1:500 dilution; Leica Biosystems, Wetzlar, Germany) overnight at 4°C. Peroxidase-conjugated goat anti-mouse IgG (Nichrei, Tokyo, Japan) was then used as a secondary antibody. The sections were then treated with 3,3-diaminobenzidine-4HCl at room temperature for 1 minute. Nuclei were then stained with Mayer's hematoxylin at room temperature for 1 minute. Images were acquired with a Keyence BZ-9000 (Osaka, Japan) bright-field microscope.

Osteoclast formation

Bone marrow-derived mononuclear cells (BM-MNCs) were isolated by density gradient centrifugation with Lymphoprep (STEMCELL Technologies, Vancouver, British Columbia, Canada) according to the manufacturer's instructions. The GM colonies enriched with osteoclast precursors were prepared by culturing BM-MNCs in Methocult H4534 Classic Without EPO (STEMCELL Technologies) for 12 days. For the osteoclast differentiation experiment, GM colonies (3×10^4 cells/well) were cultured in MEM alpha medium (Thermo Fisher Scientific, Waltham, Mass) supplemented with 10% FBS, Leukoprol (3,000 U/mL) and human soluble RANKL (30 ng/mL) in the presence or absence of IFN-γ in 96-well plates. Half of the medium was replaced every 2 days.[34,35] Cells were then subjected to molecular assays (quantitative PCR and immunoblotting) and TRAP staining after culture for 3 and 7 days, respectively. Bone marrow samples were obtained from patients with AD IFN-γR1 deficiency, AD STAT1 deficiency, or STAT1 GOF and from healthy controls after obtaining informed consent. This study was approved by the Ethics Committee/Internal Review Board of Hiroshima University. The following cytokines were used: recombinant human IFN-γ (R&D Systems, Minneapolis, Minn), recombinant human soluble RANKL (PeproTech, Rocky Hill, NJ), and Leukoprol (Kyowa Kirin, Tokyo, Japan).

Detection of differentiated osteoclasts by TRAP staining

GM colonies were cultured under osteoclast differentiation conditions for 7 days. Cells were fixed with 4% paraformaldehyde (pH 7.4) for 5 minutes and washed 3 times in distilled water. Cells were then stained for TRAP at 37°C for 30 minutes using a TRAP staining kit (Primary Cell Co, Hokkaido, Japan), washed in PBS, and dried. TRAP-positive cells were considered osteoclasts. Images were acquired with a Keyence BZ-9000 bright-field microscope. All staining images were acquired using the same exposure time.

Pit formation assay

GM colonies (3×10^4 cells/well) were seeded into 96-well plates containing $4 \times 4 \times 0.1$–mm slices of dentin (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) and cultured under osteoclast differentiation conditions (detailed above) for 14 days. The cells were removed from the dentin slices by brief sonication in 1 mol/L NH_3. The dentin slices were washed in PBS and stained with Mayer's hematoxylin solution (Fujifilm) for 1 minute. They were then washed in PBS and dried. Images were acquired with a Keyence BZ-9000 bright-field microscope.

Quantitative real-time PCR

GM colonies were cultured under osteoclast differentiation conditions for 3 days. Then, total RNA was extracted using an RNeasy Mini-Kit (Qiagen) and subjected to reverse transcription using random hexamer primers (Life Technologies, Tokyo, Japan) with a Superscript-RT Kit (Qiagen). All procedures were performed according to the manufacturer's instructions. cDNA was then subjected to quantitative real-time PCR using a StepOnePlus system (Applied Biosystems, Foster City, Calif). Data analysis was performed with the comparative computed tomography method. The following TaqMan Gene Expression Assay probes (Applied Biosystems) were used: NFATc1 (Hs00542678_m1), IRF8 (Hs00175238_m1), c-Fms (Hs00911250_m1), RANK (Hs00187192_m1), and GAPDH (Hs99999905_m1).

Immunoblot

GM-colonies were cultured under osteoclast differentiation conditions for 3 days and subjected to immunoblotting. Immunoblot analysis was performed as described in previous reports.[31,32] Briefly, cell lysates were separated by

TABLE I. The frequency of bone involvement in patients with IFN-γR1, STAT1, or IL-12Rβ1 deficiency

Disease	Inheritance and severity of the molecular defects	Patients, n/n	Frequency, %
IFN-γR1 deficiency	AR complete	18/72	25.0
	AR partial	13/23	56.5
	AD	59/83	71.1
STAT1 deficiency	AR complete	1/12	8.3
	AR partial	2/5	40.0
	AD	15/25	60.0
IL-12Rβ1 deficiency	AR	8/136	5.9

The summaries of the numbers of bone involvement are detailed in Tables E1 to E3.

10% SDS-PAGE, and proteins were transferred to polyvinylidene fluoride membranes (Merck KGaA, Darmstadt, Germany). The membranes were blocked with low-fat bovine milk. Immunoblotting was conducted with a rabbit polyclonal anti-human TRAF6 antibody (catalog sc-7221; Santa Cruz, Thermo Fisher Scientific) or mouse monoclonal anti-β-actin antibody (catalog A5316; Sigma-Aldrich, St Louis, Mo). Horseradish peroxidase–conjugated goat anti-mouse and anti-rabbit antibodies (GE Healthcare, Buckinghamshire, England) were used as secondary antibodies. Antibody binding was detected with enhanced chemiluminescence reagent (Thermo Fisher Scientific).

RESULTS

Increases in osteoclasts in the histopathology of osteomyelitis in patients with MSMD

Multifocal osteomyelitis is common in patients with MSMD with a poor IFN-γ response, such as that caused by IFN-γR1, IFN-γR2, or STAT1 deficiency.[22] Computed tomography or x-ray imaging of multifocal osteomyelitis showed osteolytic changes and concomitant bone calcification in patients with AD IFN-γR1 deficiency (patient 1) or AD STAT1 deficiency (patient 3) (Fig 1, *A*).[22,30,32] Similar findings were also observed in osteomyelitic regions in other patients with AD STAT1 deficiency carrying a heterozygous p.Y701C (patient 3's mother) or p.G250E[22,32] mutation in *STAT1* (see Fig E1 in this article's Online Repository at www.jacionline.org). Bone marrow biopsies were obtained from osteomyelitis lesions in patients 1 and 3, from 2 healthy individuals, and from osteochondroma lesions that showed abnormal osteoclast proliferation.[22,30] These sections were stained for TRAP, an osteoclast-specific marker, and examined for the presence of positive cells (Fig 1, *B*). As a positive control for TRAP staining, bone marrow biopsy tissues from osteochondroma lesions exhibited numerous TRAP-positive cells, whereas those from healthy individuals did not show TRAP-positive cells. The tissue from the osteomyelitis lesions in patients 1 and 3 showed noncaseating granulomas containing TRAP-positive giant cells. Therefore, increased osteoclasts were found in the lesions of multifocal osteomyelitis in patients with AD IFN-γR1 deficiency or AD STAT1 deficiency.

The inhibitory effect of IFN-γ on osteoclast formation and bone resorption

Osteoclasts are large, multinucleated cells that are formed by fusion of osteoclast precursor cells of the monocyte-macrophage lineage derived from hematopoietic stem cells.[36,37] GM colonies derived from BM-MNCs are osteoclast precursor cells that differentiate into osteoclasts with high efficiency in response to RANKL and M-CSF.[34,35] BM-MNCs were collected from healthy volunteers, a patient with AD IFN-γR1 deficiency (patient 1), 2 patients with AD STAT1 deficiency (patients 2 and 3), and a patient with STAT1 GOF (patient 4)[31,33] and were differentiated into GM colonies. The GM colonies were then differentiated into osteoclasts in the presence of RANKL and M-CSF, and osteoclast formation was evaluated by TRAP staining after 7 days (Fig 2, *A*). In addition, the inhibitory effect of IFN-γ on RANKL- and M-CSF–mediated osteoclast formation was examined by adding increasing concentrations of IFN-γ during differentiation. In the absence of IFN-γ, GM colonies from patients with AD IFN-γR1 deficiency, AD STAT1 deficiency, or STAT1 GOF differentiated into osteoclasts similar to GM colonies from healthy volunteers. Hereafter, these GM colonies are correspondingly referred to as AD IFN-γR1–deficient, AD STAT1–deficient, AD STAT1 GOF, and healthy cells. Osteoclast formation was almost completely inhibited by 50 IU/mL IFN-γ in healthy cells (C1-3) (Fig 2, *A*). AD STAT1 GOF cells exhibited characteristic hyperresponsiveness to IFN-γ and inhibition of osteoclast formation occurred at a lower concentration of IFN-γ (10 IU/mL) than in healthy cells. By contrast, AD IFN-γR1–deficient and AD STAT1–deficient cells required higher concentrations of IFN-γ (≥100 IU/mL) to inhibit osteoclast formation. Osteoclastogenesis requires increased expression of nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), a key transcription factor for osteoclast differentiation.[37-40] Therefore, the mRNA expression of *NFATC1*, encoding NFATc1, was examined on the third day of osteoclast differentiation induction (Fig 2, *B*). In healthy cells, *NFATC1* mRNA expression was downregulated in a concentration-dependent manner and was 50% lower in the presence of IFN-γ 50 IU/mL than in the absence of IFN-γ. In addition, in AD IFN-γR1–deficient and AD STAT1–deficient cells, the decrease in *NFATC1* mRNA was lower than that in healthy cells, with a decrease of <50% even in the presence of 50 IU/mL IFN-γ. In addition, AD STAT1 GOF cells showed a pronounced decrease in *NFATC1* mRNA expression following the addition of IFN-γ, with a decrease of >50% in the presence of a low concentration of IFN-γ (5 IU/mL). These results indicated that IFN-γ concentration-dependent inhibition of osteoclast formation is impaired in AD IFN-γR1–deficient and AD STAT1–deficient cells.

Next, the function of osteoclasts was evaluated using a pit formation assay, in which osteoclasts are formed on dentin slices for measurement of their bone-resorbing activity.[35] GM colonies were differentiated into osteoclasts in the presence of RANKL and M-CSF, and bone resorption was examined after 14 days (Fig 3). The results showed that AD IFN-γR1 deficiency and AD STAT1 deficiency showed a level of bone resorption similar to that of healthy cells. Pit formation in

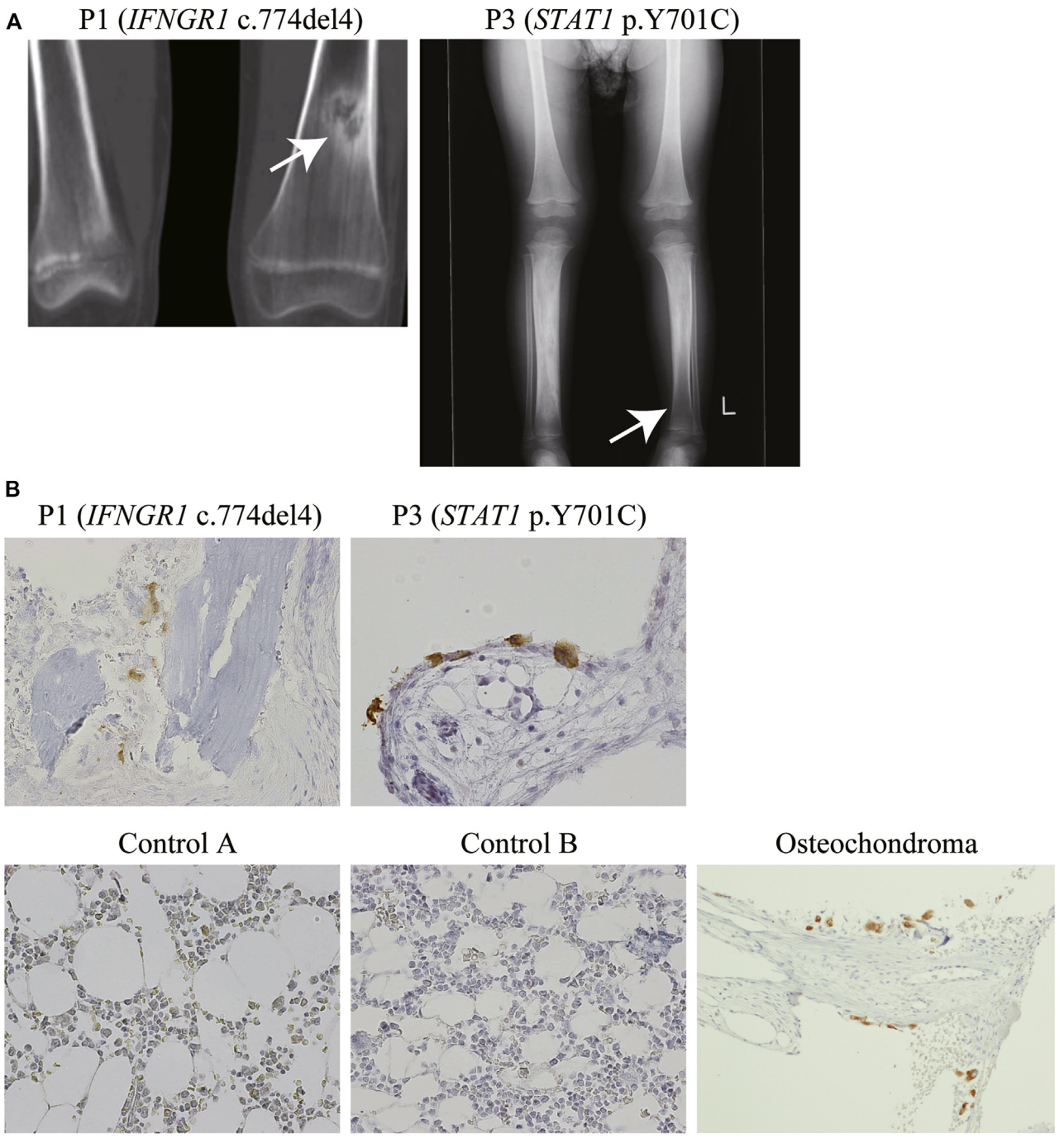

FIG 1. Increased osteoclasts in osteomyelitis lesions in patients with MSMD. **(A)** Computed tomography and x-ray images of osteomyelitis in a patient with AD IFN-γR1 deficiency (patient 1, *left*) and a patient with AD STAT1 deficiency (patient 3, *right*). Osteolytic changes and concomitant bone calcification are indicated by *white arrow*. **(B)** Immunohistochemical TRAP staining of bone marrow biopsy tissue from patients 1 and 3, healthy individuals, and a patient with osteochondroma. The histopathology of osteomyelitis in patients with AD IFN-γR1 deficiency or AD-STAT1 deficiency showed noncaseating granulomas with abundant TRAP-positive giant cells. *P*, Patient.

healthy cell cultures was strongly inhibited by 5 IU/mL IFN-γ and completely absent at 50 IU/mL. However, in AD IFN-γR1–deficient and AD STAT1–deficient cell cultures, pit formation was observed even in the presence of 100 IU/mL IFN-γ. Therefore, IFN-γ–dependent inhibition of bone resorption was impaired in AD IFN-γR1–deficient and AD STAT1–deficient cells.

Lack of IFN-γ–induced *IRF8* upregulation and *TNFRSF11A* downregulation during osteoclastogenesis

The expression of other osteoclastogenesis-related genes induced by IFN-γ. *IRF8*,[41-45] which is induced by IFN-γ stimulation and downregulated during osteoclastogenesis; c-Fms (*CSF1R*),[46] which is expressed on osteoclast precursor cells;

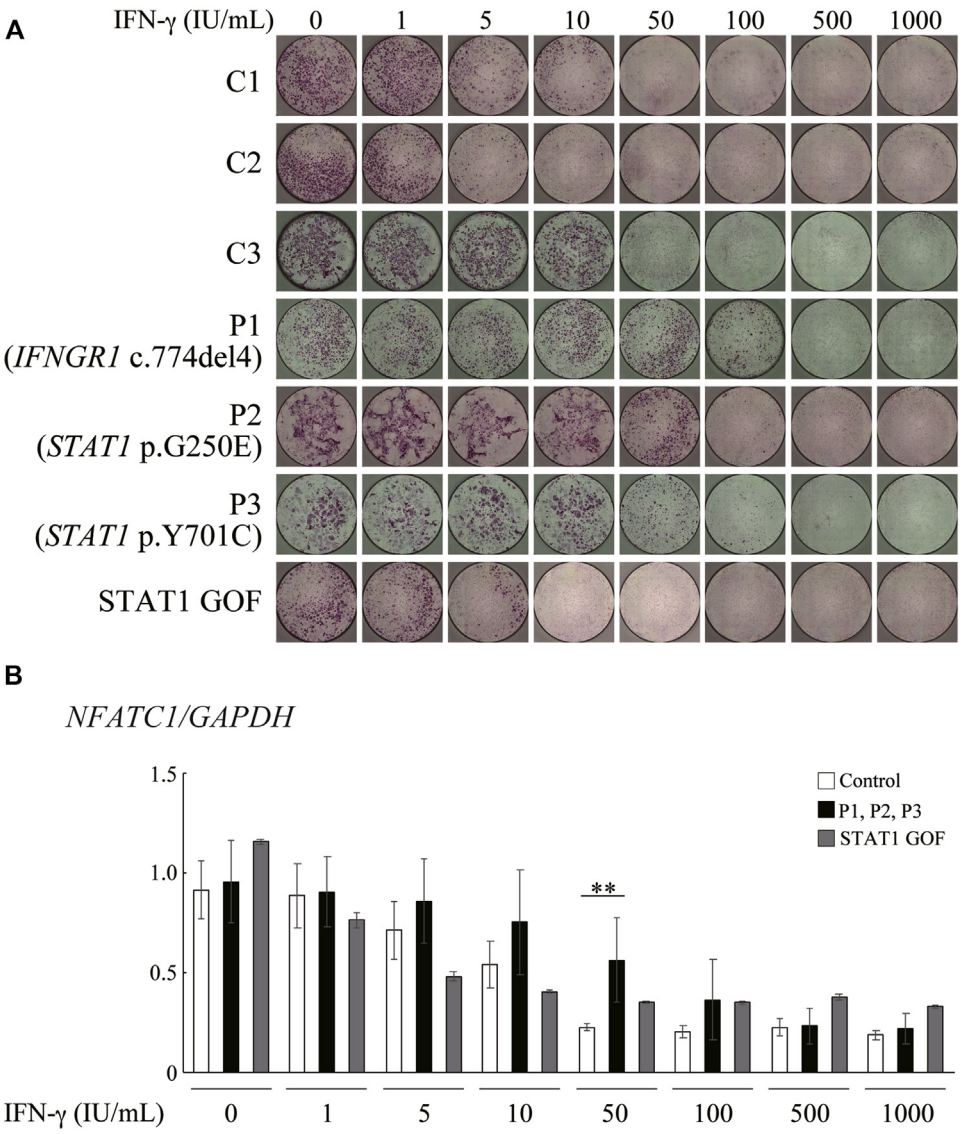

FIG 2. The inhibitory effect of IFN-γ on osteoclast formation. **(A)** GM colonies were differentiated into osteoclasts in the presence of RANKL and M-CSF, and osteoclast formation was evaluated by TRAP staining. The inhibitory effect of IFN-γ was examined by adding increasing concentrations of IFN-γ. **(B)** The dose-dependent inhibitory effect of IFN-γ on *NFATC1* mRNA expression was examined. A poor response to IFN-γ was observed in cells from patients 1 and 3. *C*, Colony.

and RANK (*TNFRSF11A*),[46-49] which is a receptor for RANKL and expressed on osteoclast precursor cells, was examined by quantitative PCR. *IRF8* mRNA was upregulated in an IFN-γ–dependent manner in all healthy, AD IFN-γR1–deficient, AD STAT1–deficient, and AD STAT GOF cells (Fig 4, *A*). *IRF8* mRNA expression was significantly lower in AD IFN-γR1–deficient and AD STAT1–deficient cells than in healthy cells at low concentrations (5-100 IU/mL) of IFN-γ. However, AD STAT1 GOF cells showed higher levels of *IRF8* mRNA expression than healthy cells under all IFN-γ stimulation conditions (Fig 4, *A*). Furthermore, *TNFRSF11A* mRNA expression was downregulated in an IFN-γ concentration-dependent manner in healthy, AD IFN-γR1–deficient, AD STAT1–deficient, and AD STAT GOF cells (Fig 4, *B*). In contrast to the findings in healthy cells, inhibition of *TNFRSF11A* mRNA expression was mild at low concentrations of IFN-γ (5-100 IU/mL) in AD IFN-γR1–deficient and AD STAT1–deficient cells but was strong at low concentrations of IFN-γ (5-50 IU/mL) in AD STAT1 GOF cells. There was no significant difference in *CSF1R* expression in response to stimulation with any concentration of IFN-γ among healthy, AD IFN-γR1–deficient, AD STAT1–deficient and AD STAT1 GOF cells (Fig 4, *C*). These results indicated that IFN-γ–induced upregulation of *IRF8* mRNA and downregulation of *TNFRSF11A* mRNA was impaired in both AD IFN-γR1–deficient and AD STAT1-deficient cells during osteoclastogenesis.

Normal TRAF6 protein expression during osteoclastogenesis

RANKL is expressed on osteoblasts and binds the receptor RANK, which is expressed on osteoclasts and their precursor cells. When osteoclast precursor cells are stimulated with

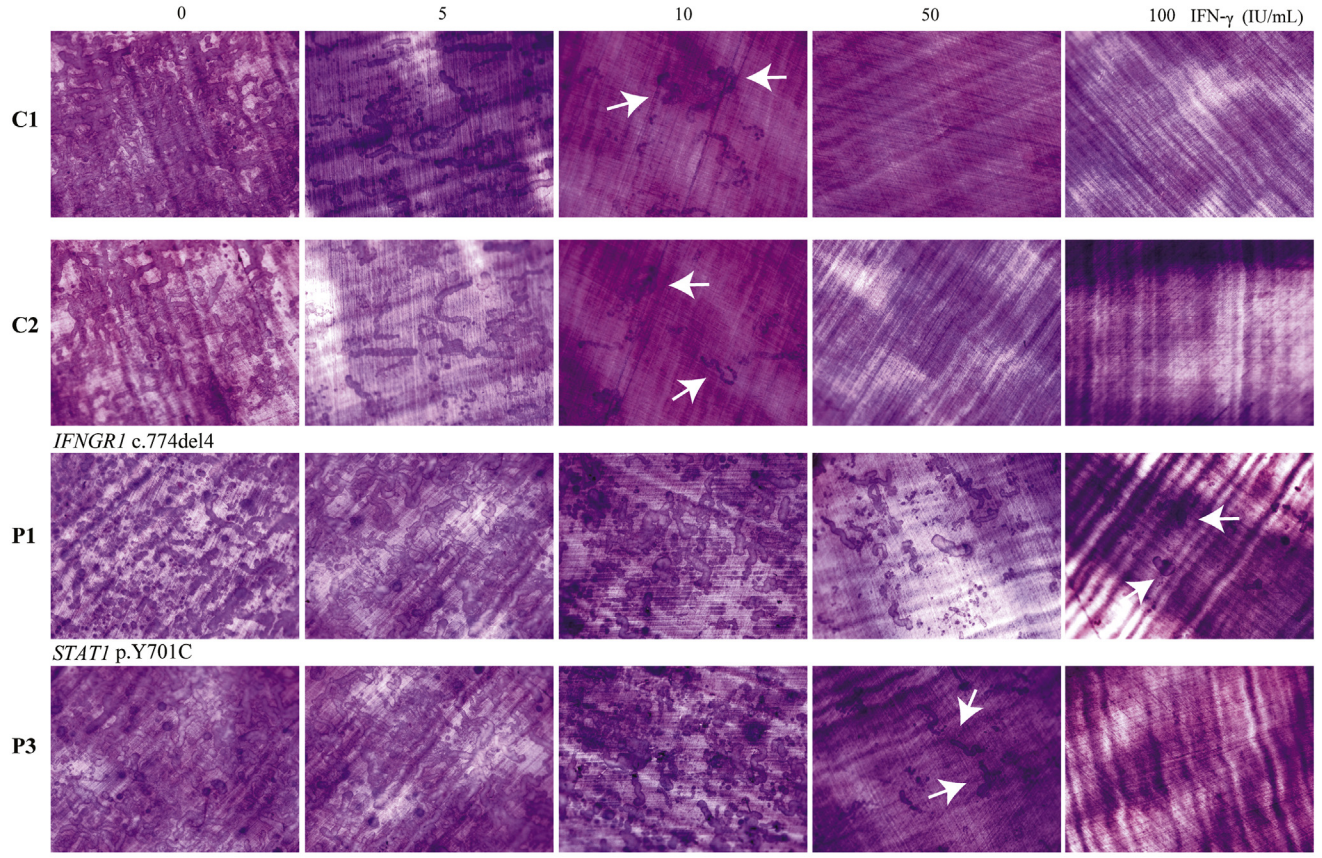

FIG 3. The inhibitory effect of IFN-γ on osteoclast bone resorption. The function of osteoclasts was evaluated using a pit formation assay. Osteoclasts from patients with AD IFN-γR1 deficiency or AD STAT1 deficiency were cocultured with increasing IFN-γ concentration with dentin slices for 14 days and imaged with bright field microscopy. Pit formation, indicated by *white arrows*, can be found in colonies 1 and 2 the condition of treated with 10 IU/mL IFN-γ (C1, C2), whereas it was found in the concentration of after treatment with 100 IU/mL and 50 IU/mL of IFN-γ in AD IFN-γR1-deficient (P1) and AD STAT1-deficient (P2) cells, respectively.

RANKL, the resulting signals are transmitted downstream through TRAF6, which promotes osteoclastogenesis.[36,37] In mice, IFN-γ has been shown to induce degradation of the TRAF6 protein via activation of the ubiquitin-proteasome system, leading to inhibition of osteoclastogenesis.[26] In humans, however, it has been suggested that IFN-γ does not induce degradation of the TRAF6 protein.[46] TRAF6 protein expression was examined at day 3 of osteoclast differentiation culture, the same time point at which *IRF8* mRNA expression was quantified (Fig 5). The results showed that TRAF6 protein expression was not decreased by IFN-γ stimulation in either healthy or AD IFN-γR1–deficient cells and suggested that IFN-γ–mediated inhibition of osteoclast formation is not transmitted via TRAF6.

DISCUSSION

Multifocal osteomyelitis is frequently observed in patients with MSMD with a poor IFN-γ response, such as that resulting from IFN-γR1, IFN-γR2, or STAT1 deficiency. In addition, there have been few reports of multifocal osteomyelitis in patients with deficiency of IL-12Rβ1 or IL-12p40, which affect both IL-12 and IL-23 signaling.[22,24,25] Given these past findings, we initiated this study to investigate the possibility that impaired IFN-γ signaling may play a role in the pathogenesis of multifocal osteomyelitis. The lesions of multiple osteomyelitis showed manifestations suggestive of osteolytic changes, and histopathological analysis of the biopsy specimens revealed an increase in multinucleated giant cells positive for TRAP, an osteoclast-specific marker. This finding suggests that there are many osteoclasts in the lesions of multifocal osteomyelitis and that bone resorption is enhanced. In the presence of RANKL and M-CSF, AD IFN-γR1–deficient and AD STAT1–deficient cells showed levels of osteoclast differentiation and bone resorption capacity similar to those of healthy cells. Osteoclast differentiation was inhibited by the addition of IFN-γ to healthy cells. This finding is consistent with those of previous studies showing that IFN-γ is a potent inhibitor of osteoclastogenesis.[26-29] By contrast, AD IFN-γR1–deficient and AD STAT1–deficient cells were resistant to IFN-γ–induced inhibition of osteoclast differentiation and bone resorption. IFN-γ–induced inhibition of *NFATC1* mRNA, a major transcription factor for osteoclast differentiation, was also impaired in AD IFN-γR1–deficient and AD STAT1–deficient cells. These results suggest that impairment of IFN-γ–induced inhibition of osteoclast differentiation and bone resorption in the context of both deficiencies,

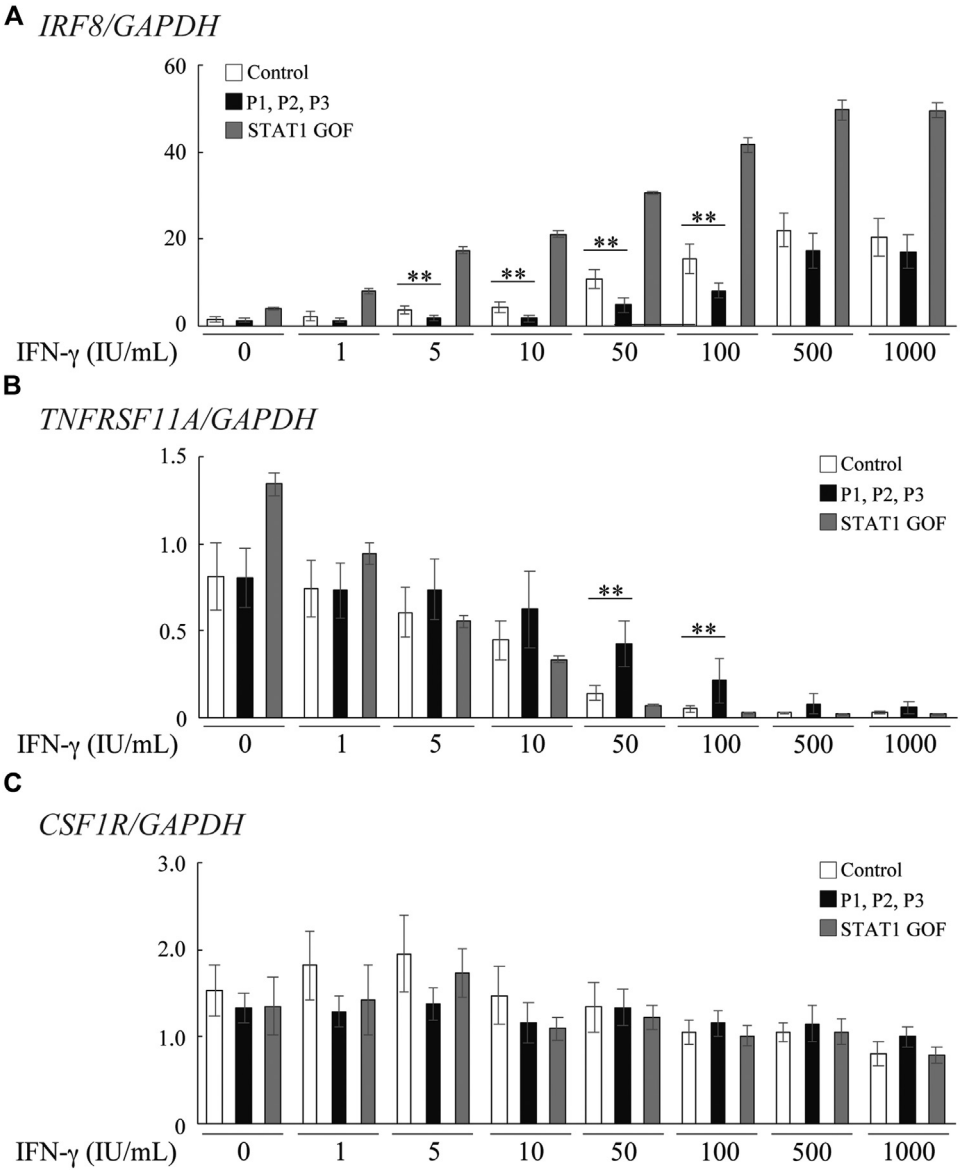

FIG 4. The effect of IFN-γ on gene expression during osteoclastogenesis. GM colonies from patients with AD IFN-γR1 deficiency or AD STAT1 deficiency were treated with increasing concentrations of IFN-γ for 3 days. Quantitative PCR measurement of *IRF8* **(A),** *TNFRSF11A* **(B),** and *CSF1R* **(C)** mRNA expression during osteoclastogenesis was performed at day 3.

leading to excessive osteoclast proliferation and increased bone resorption in infection foci, may underlie multifocal osteomyelitis.

Compared with healthy cells, AD IFN-γR1–deficient and AD STAT1–deficient cells showed a decrease in *IRF8* mRNA expression and an increase in *TNFRSF11A* mRNA expression following the addition of IFN-γ to the osteoclast differentiation culture medium. However, transcriptional regulation of *CSF1R* after the addition of IFN-γ was similar in healthy cells and patient cells (AD IFN-γR1–deficient, AD STAT1–deficient and AD STAT1 GOF cells). It has been demonstrated that IRF8 acts as a negative regulator of osteoclast differentiation and that its repression leads to the promotion of osteoclastogenesis.[42,45,50] Therefore, IRF8-deficient mice develop osteoporosis due to enhanced osteoclast differentiation.[45] In addition, the development of multiple idiopathic root resorption, a type of periodontal disease, and accelerated osteoclast formation have been reported in patients carrying a homozygous p.G388S mutation in *IRF8*, which impairs the ability to transmit signals to NFATc1.[51] This finding also suggests that IRF8 may play an important role in regulating bone resorption at the site of inflammation. Because IFN-γ induces IRF8 via STAT1 activation, induction of IRF8 was inhibited in the context of both deficiencies with an impaired IFN-γ response, which tended to promote osteoclast formation. The expression of *TNFRSF11A* mRNA in healthy cells was inhibited with the addition of IFN-γ under osteoclast differentiation conditions. This finding is consistent with those of previous reports that IFN-γ treatment induced methylation of the promoter region of the *TNFRSF11A* gene and inhibited its expression in macrophages.[49] Inhibition of *TNFRSF11A* mRNA expression by

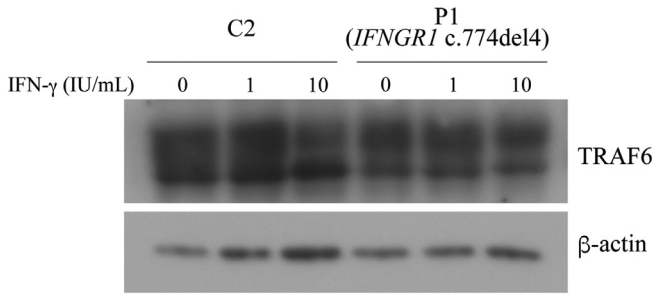

FIG 5. TRAF6 protein expression during osteoclastogenesis. GM colonies from a health donor and a patient AD IFN-γR1 deficiency were differentiated into osteoclasts in the presence of RANKL and M-CSF, followed by IFN-γ treatment for 3 days. TRAF6 protein expression was evaluated by immunoblotting.

IFN-γ was impaired in AD IFN-γR1–deficient and AD STAT1–deficient cells. RANK, which induces NFATc1 expression in response to RANKL stimulation and leads to the differentiation of mature osteoclasts, is mainly expressed on osteoclast precursor cells.[37] Therefore, mice lacking RANK or RANKL exhibit osteopetrosis due to osteoclast hypoplasia.[47] Moreover, GOF mutations in *TNFRSF11A*, affecting the RANK signal peptide, have been shown to cause familial expansile osteolysis via hyperactivation of osteoclasts.[52] Therefore, the IFN-γ–induced impairment of RANK inhibition in AD IFN-γR1–deficient and AD STAT1–deficient cells may also be related to the promotion of osteoclast formation and osteoclast hyperactivation.

In the present study, we demonstrated a link between enhanced osteoclast differentiation due to diminished inhibition by IFN-γ and multifocal osteomyelitis in patients with MSMD with an impaired response to IFN-γ. The occasional detection of acid-fast bacilli at bone biopsy specimens suggests that infection might be a trigger of the osteomyelitis. On the other hand, chronic nonbacterial osteomyelitis (CNO) is found in autoinflammatory disorders, such as pyogenic arthritis, pyoderma gangrenosum, and acne syndrome; deficiency of the IL-1-receptor antagonist; Majeed syndrome, synovitis, acne, pustulosis, hyperostosis, and osteitis syndrome; and chronic recurrent multifocal osteomyelitis.[53] The pathophysiology of CNO is incompletely understood. However, increased inflammatory cytokines (IL-1, IL-6, TNF-α, and IL-20) that enhance osteoclast differentiation and activation through upregulation of RANKL-RANK signaling are supposed to be involved in the pathophysiology of CNO.[54] The bone involvement in patients with MSMD and autoinflammatory disorders may share pathophysiological aspects in enhanced differentiation and activation of osteoclasts, although the suspected mechanisms (eg, impairment of inhibition of osteoclastogenesis by poor response to IFN-γ vs enhanced osteoclastogenesis associated with increased inflammatory cytokines) underlying this observation are different. Because bisphosphonate directly inhibits RANKL-stimulated osteoclast differentiation and is used for the treatment of CNO, it might be effective in the treatment of patients with MSMD with osteomyelitis.[54,55]

Our study has several limitations. First, we did not analyze GM colonies derived from patients with deficiency of IL-12Rβ1 or IL-12p40, which are involved in impaired IL-12 and IL-23 signaling. Because the response to IFN-γ is maintained in diseases associated with both deficiencies, the study of osteoclast differentiation in cells from patients with these types of deficiencies may provide important insights into the pathogenesis of multifocal osteomyelitis in patients with MSMD. Second, we were not able to analyze comprehensive gene expression profiles during osteoclast differentiation. These limitations are solely due to the rarity of MSMD. In fact, neither IL-12Rβ1 nor IL-12p40 deficiency has been reported in Japan. In addition, it is difficult to obtain bone marrow–derived cells from patients with MSMD that do not show abnormalities in the hematopoietic system. Despite these limitations, we provided valuable information for considering the association between impaired IFN-γ–mediated signaling and the development of multifocal osteomyelitis. However, there still are many questions for multifocal osteomyelitis in patients with MSMD. The frequency of bone involvement is high in AD IFN-γR1 deficiency or AD STAT1 deficiency. In contrast, it is relatively low in AR complete IFN-γR1 deficiency or AR complete STAT1 deficiency (Table I, Tables E1-E3), although patients with these 2 disorders show completely abolished cellular response to IFN-γ. The mechanism of this observation is uncharacterized, but we suspect 2 possibilities to explain it. First, histopathological analysis of biopsy specimens of osteomyelitis in patients with MSMD generally shows granuloma, suggesting the presence of chronic inflammation, but in the patients with AR complete IFN-γR1 deficiency or AR complete STAT1 deficiency severely impaired granuloma formation is shown.[2,56,57] Second, both AR complete IFN-γR1 deficiency and AR complete STAT1 deficiency are life-threatening and early intervention with hematologic stem cell transplantation is necessary for survival.[58,59] The severity of these disorders makes long-term observation of the clinical course difficult, potentially leading to an underestimation of the frequency of bone involvement. The mechanism underlying multifocal osteomyelitis without other organ involvement in patients with AD IFN-γR1 deficiency and AD STAT1 deficiency is also uncharacterized.[19,32] The collection of more patients and scientific validation of results is necessary to understand the entire molecular pathogenesis of multifocal osteomyelitis in patients with MSMD.

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Key messages

- **IFN-γ concentration-dependent inhibition of osteoclast formation is impaired in GM colonies from patients with AD IFN-γR1 deficiency or AD STAT1 deficiency.**
- **Excessive osteoclast proliferation and increased bone resorption at infection foci may lead to multifocal osteomyelitis in patients with MSMD due to an impaired response to IFN-γ.**

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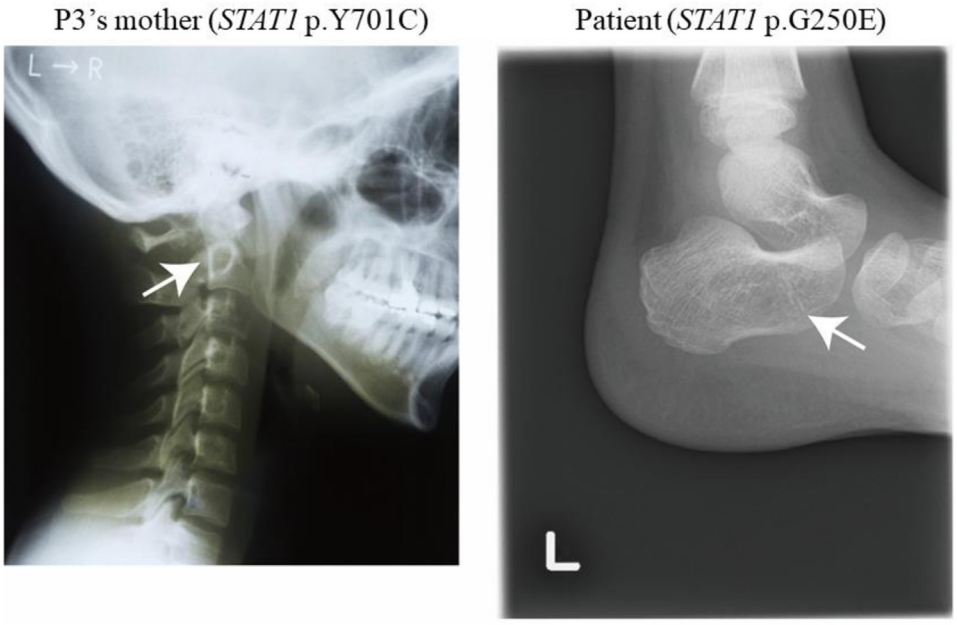

FIG E1. X-ray images of osteomyelitis in patients with MSMD. X-ray images of multifocal osteomyelitis showed osteolytic changes and concomitant bone calcification *(white arrow)* in patients with AD STAT1 deficiency carrying a heterozygous p.Y701C (patient 3's mother) (*left*) or p.G250E (*right*) mutation.

TABLE E1. The frequency of bone involvement in patients with IFN-γR1 deficiency

	Cases reported	Bone involvement	References
AR complete	59	16	E1-E14
	1	0	E15
	3	2	E16
	8	0	E17
	1	0	E18
	72	18	18/72 (25.0%)
AR partial	20	10	E1, E4, E19-E22
	2	2	E15
	1	1	E23
	23	13	13/23 (56.5%)
AD	68	49	E11, E24-E44
	1	1	E3
	1	1	E45
	1	1	E46
	1	1	E47
	1	0	E48
	2	1	E49
	1	0	E50
	7	5	E17
	83	59	59/83 (71.1%)

TABLE E2. The frequency of bone involvement in patients with STAT1 deficiency

	Cases reported	Bone involvement	References
AR complete	4	0	E51
	1	0	E52
	1	0	E53, E54
	2	0	E55
	1	1	E56
	1	0	E57
	2	0	E17
	12	1	1/12 (8.3%)
AR partial	2	1	E58
	2	0	E59
	1	1	E60
	5	2	2/5 (40.0%)
AD	12	6	E1, E61-E65
	8	6	E66
	1	1	E67
	2	2	E16
	2	0	E17
	25	15	15/25 (60.0%)

TABLE E3. The frequency of bone involvement in patients with IL-12Rβ1 deficiency

	Cases reported	Bone involvement	References
AR	30	2	E4, E17, E68
	19	2	E48, E69, E70
	5	1	E45, E71, E72
	18	0	E73
	1	0	E74
	3	0	E75
	1	0	E76
	1	0	E77
	4	0	E78
	1	0	E79
	1	1	E80
	10	0	E81
	1	0	E82
	22	0	E16
	3	0	E83
	1	0	E84
	6	0	E18
	9	2	E85
	136	8	8/136 (5.9%)

In addition to the above cases, the summary of 180 cases with AR IL-12Rβ1 deficiency was reported in the previous study.[E1] To be strict, this report was excluded in this study because there was no description about bone involvement. Therefore, the data shown in this table may overestimate the frequency of bone involvement.