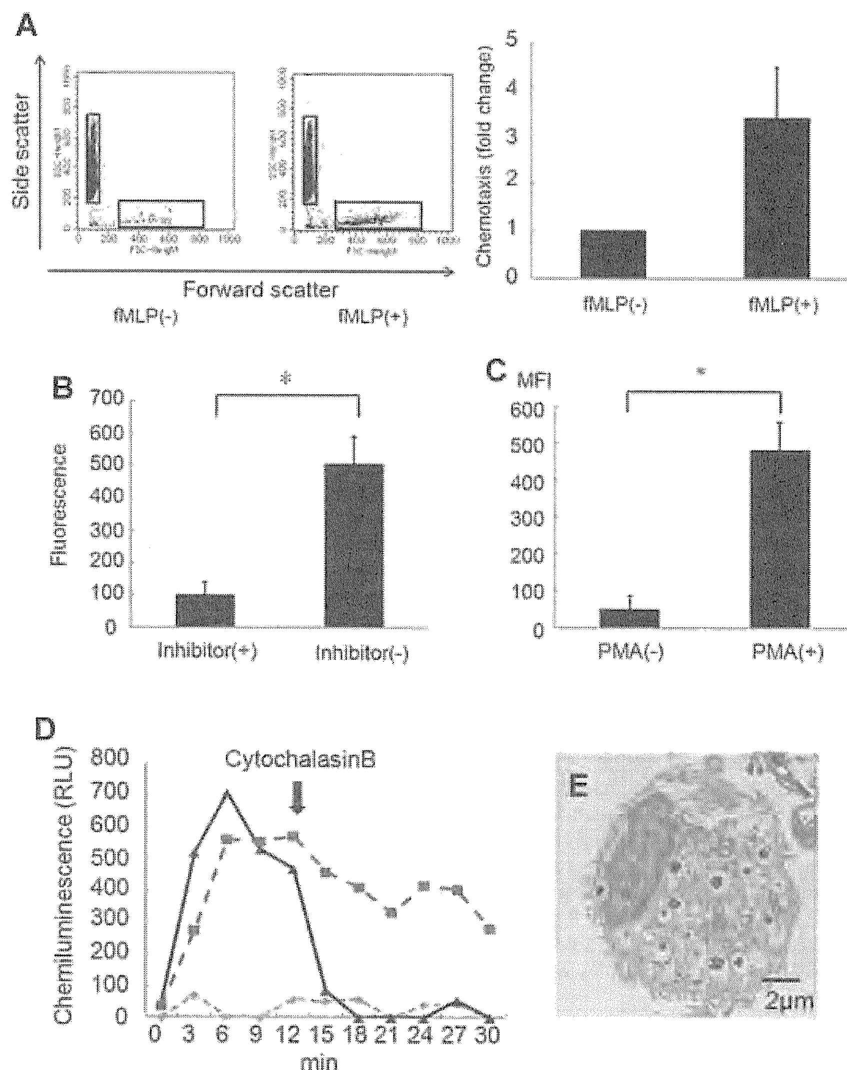


**Fig. 3. Functional analysis of hiPSC-derived neutrophils. (A) Chemotactic activity of floating cells on day10 + 30 in response to fMLP was determined as described in Materials and Methods section. After a 4-h culture, the transwell inserts were removed, and the cells in the lower chamber were counted by an LSR flow cytometer (n = 3; bars represent SDs). (B) MPO chlorination activity in cell lysates from floating cells on day10 + 30 was analyzed by EnzChek Myeloperoxidase (MPO) Activity Assay Kit as described in the Materials and Methods section. The chlorination activity in neutrophil cell lysates was almost completely abolished by the addition of a chlorination inhibitor (n = 3; bars represent SDs; $^*P < 0.05$.). (C) Floating cells on day10 + 30 were subjected to DHR assay. DHR was reacted with neutrophils with or without PMA, and the resultant rhodamine fluorescence was detected by flow cytometry. The addition of PMA increased the levels of fluorescence. Results are expressed as mean fluorescence intensity (MFI) (n = 3; bars represent SDs; $^*P < 0.05$.). (D) Floating cells on day10 + 30 were subjected to the assay for phagocytosis-induced respiratory burst activity using chemiluminescent microspheres (luminol-binding microspheres). Gradual increase in chemiluminescence indicates the respiratory burst triggered by the phagocytosis of luminol-binding microspheres (squares). The increase in chemiluminescence was almost completely abolished by the addition of cytochalasin B (diamonds) and inhibited by its later addition (triangles). The figures are representative of three independent experiments. Abbreviation: RLU, relative light units. (E) hiPSC-derived neutrophils phagocytosing the microbeads were analyzed by transmission electron microscopy.**

differentiation in this culture system were investigated by RT-PCR (Fig. 4E–F). NANOG, a pluripotency marker, was expressed in undifferentiated iPS cells but disappeared in sorted VEGFR2$^{high}$CD34$^{+}$ cells after 10 days differentiation. PU.1 and C/EBPα, essential transcription factors for commitment and differentiation of the granulocytic lineage (Borregaard et al., 2001; Friedman, 2007) were first detected on day 10 + 10 and persisted thereafter. C/EBPε, which had a critical role for the later stages of neutrophil development and transcription of key granule proteins (Borregaard et al., 2001; Friedman, 2007) were first detected faintly on day 10 + 10 and upregulated thereafter. MPO and lactoferrin, which were expressed at the highest levels in myeloblasts/promyelocytes and myelocytes/metamyelocytes, respectively (Cowland and Borregaard, 1999; Borregaard et al., 2001), were detected on day 10 + 10. Gelatinase, which was expressed at the highest level in band and segmented neutrophilic cells (Cowland and Borregaard, 1999; Borregaard et al., 2001), was first detected on day 10 + 20 and upregulated thereafter. Altogether, these results suggested that the neutrophil differentiation in this co-culture system might recapitulate the orderly differentiation process in bone marrow.

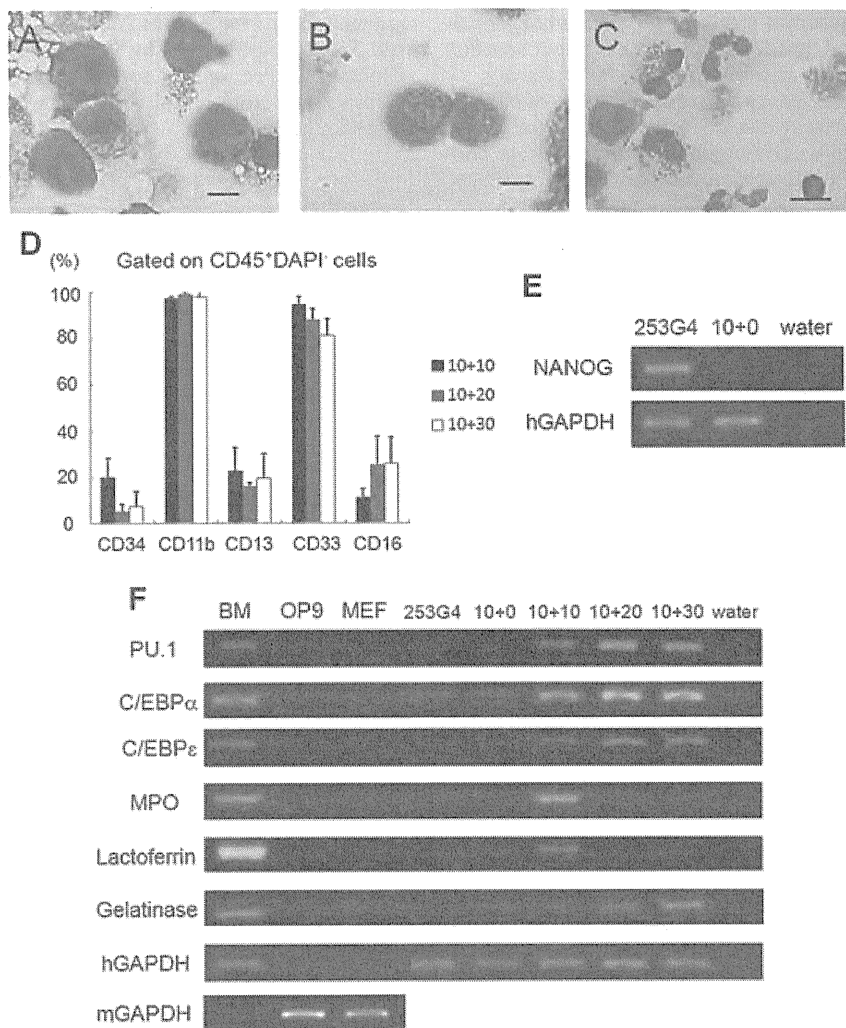


**Fig. 4. Sequential analysis of neutrophil differentiation from hiPSCs. (A–C) Sequential morphological analysis of day 10 + 10 (A), day 10 + 20 (B) and day 10 + 30 (C). Scale bars: 10 μm. (D) Surface antigen expression at each level of differentiation of hiPSC-derived cells was analyzed by flow cytometry. All adherent cells including OP9 cells were harvested and stained with antibodies. Human $CD45^{+}DAPI^{-}$ cells were gated as hiPSC-derived viable leukocytes (n = 3; bars represent SDs). (E–F) Sequential RT-PCR analysis of a pluripotency marker (E), genes associated with neutrophil development and neutrophils-specific granules (F) during differentiation. Human GAPDH was used as a loading control. Abbreviations: BM, human bone marrow cells; 253G4, undifferentiated 253G4 cells; 10 + 0, sorted $VEGFR2^{high}CD34^{+}$ cells after 10 days differentiation; 10 + 10, 20, 30, all cells after 10, 20, 30 days differentiation after cell sorting; hGAPDH, human GAPDH; mGAPDH, mouse GAPDH. The figures are representative of three independent experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]**

## Discussion

The analysis of the differentiation process of neutrophils can provide helpful information for the elucidation of the pathogenesis of hematopoietic diseases that affect neutrophils and/or myeloid differentiation, including inherited bone marrow failure syndromes and neutrophil function disorders. Traditionally, HL-60, an acute promyelocytic cell line, has been used as a neutrophil differentiation model (Collins et al., 1978; Newburger et al., 1979). Although this cell line grows well and differentiates easily into neutrophils, the neutrophil differentiation model is not suitable for the analysis of neutrophil-affected disorders because of its leukemic cell-origin. Development of a neutrophil differentiation system based on iPS cells would provide a better model for the analysis of such diseases, because iPS cells can be generated from the somatic cells of patients suffering from these diseases.

The current study aimed to investigate two issues in hiPSC-derived neutrophil differentiation: tracking the step-wise maturation in vitro and evaluating the wide spectrum of neutrophil functions. Through the use of a modified OP9 co-culture system, the directed and step-wise differentiation from hiPSCs to mature neutrophils containing neutrophil specific granules was first accomplished. The expression of surface antigens, transcription factors and granule proteins during differentiation exhibited the characteristic pattern of normal granulopoiesis. The biological functions of hiPSC-derived neutrophils were demonstrated through the quantitative assessment of granule enzyme activities and biological bactericidal activities such as chemotaxis and phagocytosis.

Defects in the maturation and function of neutrophils are associated with certain blood diseases including inherited bone marrow failure syndromes and neutrophil function disorders.

Among bone marrow failure syndromes, certain conditions affect a specific maturation stage, such as the maturation arrest at the plomyelocyte/myelocyte stage seen in severe congenital neutropenia. Neutrophil function disorders can affect specific bactericidal activities, such as the absence of MPO activity characteristic of MPO deficiency disorders. The use of hiPSCs for the investigation of these diseases requires sequential analyses that can identify each neutrophil maturation stage and include a functional analysis to evaluate each bactericidal activity separately on disease-specific, iPSC-derived neutrophils. Although previous studies have reported neutrophil differentiation models from hESCs (Choi et al., 2009; Saeki et al., 2009; Yokoyama et al., 2009) and hiPSC-derived neutrophils have been shown before (Choi et al., 2009), evidence showing that hiPSCs, which are artificially reprogrammed somatic cells, can follow the normal developmental pathway into fully functional mature neutrophils is of great significance, and the description of methods for identifying each neutrophil maturation step and analyzing each bactericidal pathway separately is important for clinical applications.

Although flow-cytometric analysis combined with RT-PCR identified the neutrophil maturation step relatively successfully, discrepancies between the neutrophil differentiation system in this study and normal granulopoiesis were noted such as the lower expression of CD16 than that shown by previous reports on hESC-derived neutrophils (Choi et al., 2009; Saeki et al., 2009; Yokoyama et al., 2009). As CD16 is a mature neutrophil marker in peripheral blood, two reasons could explain this phenomenon. First, residual precursors could have been more significant contaminants in the present system than in previously reported methods due to the function of cytokines and stroma supporting immature hematopoietic cells. Another possible reason is the shift of protein types between membrane-bound and soluble forms. Calluri previously reported that G-CSF is not only a myeloid cell growth factor, but also a modulator of neutrophil behavior (Carulli, 1997), and its stimulation decreases the membrane bound CD16 and increases its soluble form. Low CD16 expression has been documented in neutrophils derived in vitro from bone marrow $CD34^+$ cells by stimulation with G-CSF (Kerst et al., 1993b), and it has been observed in vivo when G-CSF is administered to healthy volunteers (Kerst et al., 1993a). This phenomenon, which is also documented in a report of hESC-derived neutrophils (Yokoyama et al., 2009), is unavoidable in differentiation culture systems using recombinant cytokines. The combination of flow cytometric and PCR analyses enables a more accurate staging of progenitors that could be of importance in the investigation of maturation arrest in future studies.

The culture system presented in this study is considered ineligible for clinical applications due to the use of xenogeneic factors such as OP9 cells and FCS. To overcome this problem, a xeno-free hematopoietic differentiation system from pluripotent cells is currently being established.

In conclusion, the present study shows the establishment of a fully functional mature neutrophil differentiation system from hiPSCs and the detailed analysis of their function and differentiation process. This system could become a useful tool for the investigation of various hematological diseases with defects in maturation and function of neutrophils.

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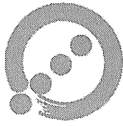



Review Article

# Autoinflammatory diseases - a new entity of inflammation

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The autoinflammatory diseases are characterized by seemingly unprovoked episodes of inflammation, without high-titer autoantibodies or antigen-specific T cells. The concept was proposed ten years ago with the identification of the genes underlying hereditary periodic fever syndromes. NLRP3 inflammasome activation and IL-1β secretion have recently emerged as a central mechanism in the pathogenesis of disease. Here we describe four genetically defined syndromes like cryopyrin-associated periodic syndromes (CAPS, cryopyrinopathies), mevalonate kinase deficiency (MKD) or hyper-IgD and periodic fever syndrome (HIDS), pyogenic aseptic arthritis, pyoderma gangrenosum, and acne syndrome (PAPA syndrome), and deficiency of interleukin-1-receptor antagonist (DIRA) along with the pitfall for understanding the pathphysiology.

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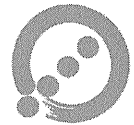
**Key words:**
autoinflammatory disease, NLRP3 inflammasome, IL-1β, cryopyrin-associated periodic fever (CAPS), mosaicism



## Introduction

Inflammation has evolved as a physiologic mechanism necessary to defend our bodies from external and internal danger triggers such as infectious agents, chemical factors, and physical factors [1].

The innate immune system is assigned to recognize and encounter these stimuli. Recently, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) have emerged as key players for the proper accomplishment of this process through recognition of pathogen associated molecular patterns (PAMPs) [2]. In addition to PAMPs, NLRs also sense endogenous stress signals known as damage associated molecular patterns (DAMPs) [2,3]. NLR dependent recognition of either exogenous or endogenous danger signals

initiates the recruitment of adaptor proteins and the formation of molecular platforms referred to as inflammasomes[2, 3]. In other word, inflammasomes are cellular alarts that assemble in response to microbial invasion and/or cellular damage and alert the system by triggering an inflammatory response. The subsequent activation of caspase-1 results in the post-transcriptional, proteolytic modulation of the related cytokines interleukin-1β (IL-1β) and IL-18 from their precursor to their active and secreted form, enhancing the inflammatory process. Among severalNLRs that form inflammasome platforms, the most studied are NALP1,NALP3 (NLRP3) and IPAF [2, 3, 4].

The identification of the critical role of NLRP3 inflammasome in the maturation of these inflammatory cytokines prompted the study of its role in the pathogenesis of several syndromes. The term IL-1β dependent autoinflammatory syndromes has been adopted for such syndromes. This group of diseases is characterized by defective regulation of innate immune response and the absence of autoantibodies or antigen-specific T cells [5].

Dysregulation of NLRP3 inflammasome based on mutations of inflammasome related genes has been implicated in the pathogenesis of cryopyrin-associated periodic fever syndrome (CAPS), hyper-IgD syndrome (HIDS), pyogenic arthritis, pyoderma gangrrenosum, and acne syndrome (PAPA), or deficiency of IL-1 receptor antagonist (DIRA) [6]. Interestingly, NLRP3 inflammasome activation by danger signals such as monosodium urate (MSU), calcium pyrophosphate dehydrate (CPPD), amyloid-beta, glucose or silica and asbestos is proposed as a key molecular mechanism in diseases including gout, pseudogout, Altheimer's disease, pulmonary fibrosis or the 2 diabetes mellitus [5].

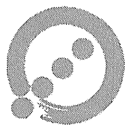
We discuss in this review about this new-coming entity of diseases along with the pitfall for understanding the pathphysiology.

## NLRP3 inflammasome

Recognition of microorganisms by the innate immune system depends on conserved germ line-encoded receptors called pattern-recognition receptors (PRRs) that sense conserved motifs present on microbes named PAMPs [7].

PRRs are classified into three groups: secreted, trans-membrane and cytosolic (Fig. 1). Secreted PRRs such as collectins, ficolins and pentraxins bind microbes and activate the complement system. The trans-membrane PRRs are Toll-like receptors (TLRs) and the C-type lectins, with some members expressed on cell surface (such as TLR2/4 and Dectin1/2) and some expressed on endosome membrane (TLR3/7/9). The cytosolic PRRs include the RIG-I-like receptors (RLRs), the nucleotide-binding domain leucine-rich repeat containing receptors (NLRs) and the newly identified DNA sensors AIM2 (absent in melanoma 2) and IFI16 (interferon-inducible protein16) [8,9,10]. Although the RLRs mainly detect viral pathogens, the NLRs can detect both PAMPs and DAMPs [11]. In response to PAMPs or DAMPs, a subset of NLRs forms a complex with ASC (apoptosis-associated speck-like protein containing a CARD) to activate caspase-1 [12]. In 2002, Tschopp group first named this complex the inflammasome [13]. Up to date, at least 4 different inflammasomes have been identified; they are the NLRP1, NLRP3, IPAF (NLRC4) and AIM2 inflammasomes [14].

NLRP3, also called CIAS1, PYPAF1, Cryopyrin, CLR1.1 (CATERPILLAR 1.1) or NALP3, is one of the best characterized NLR family members. In mice, NLRP3 is mainly expressed in tissues such as lung, liver, kidney, colon and ovary, with particularly high expression in the skin and eye [15, 16]. Mouse neutrophils, peripheral blood mononuclear cells (PBMCs) and bone marrow-derived dendritic cells (BMDCs) express high level of NLRP3 constitutively, while the bone marrow- derived macrophages (BMDMs) and Th2 cells only express this molecule at moderate level [15, 16]. However, upon TLR or TNF receptor stimulation, the expression of NLRP3 in BMDMs is dramatically elevated, largely as a result of NF-κB





activation [16, 17]. Both mouse and human osteoblasts express NLRP3 [18]. In addition, primary human PBMCs, the monocyte-derived THP-1 cell line, primary human keratinocytes (PK), keratinocyte-derived HaCaT cells, primary mast cells (MS), granulocytes and B cells all express NLRP3 [19, 20, 21]. The tissue distribution of human NLRP3 is also found in the urothelial layer in the bladder and in epithelial cells lining the oral and genital tracts besides the skin cells mentioned above [20,21].

secreted
- collectin
- ficolin
- pentraxin

trans-membrane
- Toll-like receptor (TLR)
- C-type lectin

cytosolic
- RIG-I-like receptor (RIR)
- nucleotide-binding domain leucine-rich repeat containing receptor (NLR)
- absent in melanoma 2 (AIM2)
- interferon-inducible protein 16 (IFI16)

**Fig. 1 Classification of pattern-recognition receptors**

Recognition of microorganisms by the innate immune system depends on conserved germ line-encoded receptors called pattern-recognition receptors (PRRs) that sense conserved motifs present on microbes named PAMPs.
PRRs are classified into three groups: secreted, trans-membrane and cytosolic.

Structural analysis revealed that NLRP3 contains an N-terminal pyrin domain, an intermediate NACHT domain and a C-terminal LRR domain. Upon activation,NLRP3 recruits ASC via a pyrin-pyrindomain interaction and the recruited ASC binds to pro-caspase-1 via a CARD–CARD interaction. The multiprotein complex thus formed, now called the NLRP3 inflammasome, then activates caspase-1, and the latter cleaves pro-interleukin-1β (IL-1β) and pro-IL-18 to form mature IL-1β and IL-18, respectively (Fig. 2) [22]. Whole pathogens, PAMPs, DAMPs and environmental irritants can all activate the NLRP3 inflammasome. However, the exact mechanism(s) leading to NLRP3 inflammasome activation is still not clear. Given the diversity of these NLRP3 activators, a consensus is emerging that there is a common downstream intracellular activator that constitutes a final common pathway for NLRP3 activation (Fig. 2) [23,24]. In any case, the mature IL-1β and IL-18 production resulted from NLRP3 activation are highly potent proinflammatory mediators important for host defense against infectious agents. In addition, via IL-1β, the NLRP3 inflammasome is linked to Th17 cell differentiation [25,26] as well as to Th2 response since vaccination with aluminum adjutants also activates this inflammasome [27,28]. It should be noted, however, that NLRP3-mediated secretion of IL-1β and IL-18 must be under tight control, as excessive production of these cytokines can lead to autoinflammatory diseases. This is seen in the group of diseases collectively called cryopyrin(CIAS1, NLRP3)-associated periodic syndromes (CAPSs) which are caused by hyper-activation of the NLRP3 inflammasome due to mutations in the NLRP3 gene [29,30]. Besides, hyperimmunoglobulinemia D with periodic fever syndrome (HIDS), the deficiency of the IL-1 receptor antagonist (DIRA), and the syndrome of pyogenic arthritis with pyoderma gangrenosum and acne (PAPA) are caused by mutations in genes encoding proteins that directly or

indirectly correlate NLRP3 [8].

## Cryopyrin-associated periodic syndrome (CAPS) or cryopyrinopathies

The cryopyrin-associated periodic syndrome spectrum, which encompass FCAS(Familial cold autoinflammatory syndrome), MWS(Muckle-Wells syndrome), and NOMID/CINCA syndrome (Neonatal onset multisystem inflammatory disease/chronic infantile neurologic, cutaneous, and articular syndrome), is caused by mutations in the cold induced autoinflammatory syndrome 1 (CIAS1) gene, first identified in 2001 [31]. CIAS1 codes for the protein cryopyrin, also known as NLRP3 or PYPAF1 [32]. We use the term NLRP3 thereafter. The cryopyrinopathies are transmitted in an autosomal dominant pattern. The NLRP3 gene is located on chromosome 1q44 and has 9 exons. Roughly 85% of NLRP3 mutations occur in exon 3 [29, 33]. Clinical manifestations vary among the three cryopyrinopathies, but several common features are often found, such as fever, pseudourticarial rash, joint involvement, and profoundly elevated inflammatory markers [32]. The most consistent finding across the CAPS spectrum is a migratory, maculopapular, urticaria-like, and usually nonpruritic rash. Skin biopsy reveals polymorphonuclear perivascular infiltration of the dermis, which contrasts with the biopsy findings of classical urticaria. The unique features of each of the cryopyrinopathies are described below [34].

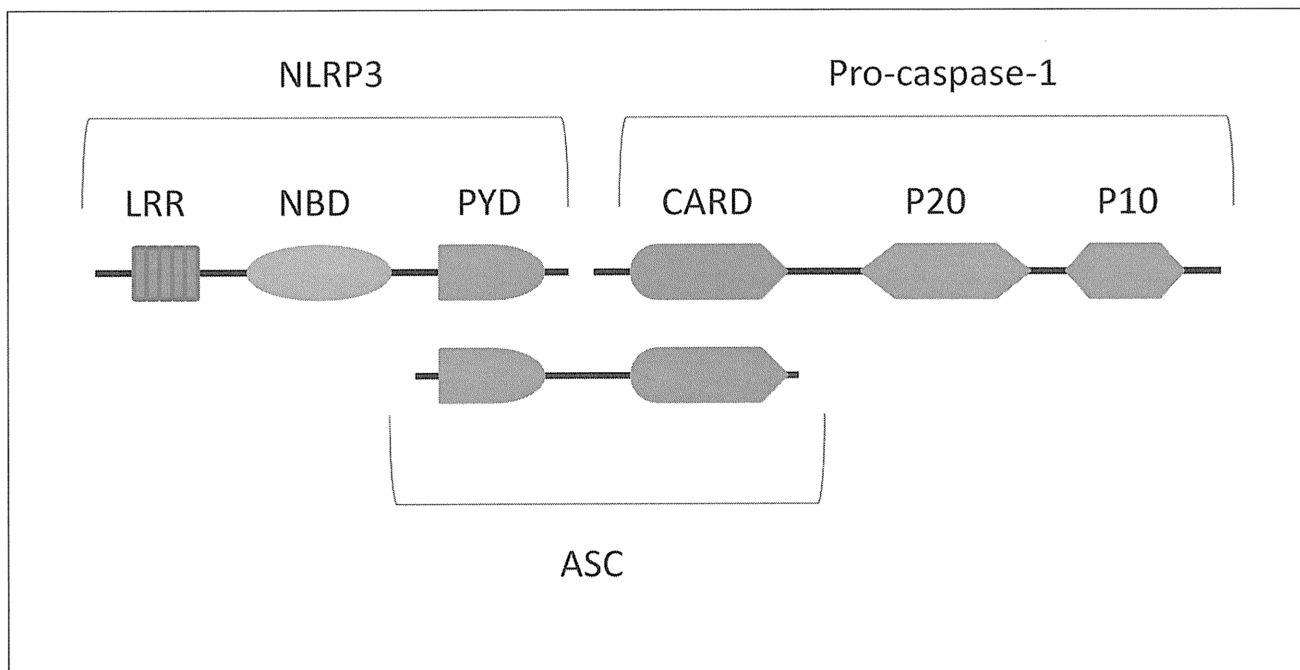
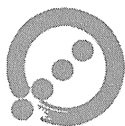



**Figure 2 Schematic structure of NLRP3 inflammasome**

Activation leads to the binding of NLRP3 with ACS through PYD-PYD interaction, resulting in recruiting of pro-caspase-1 via CARD-CARD interaction.
LRR: leucine-rich –repeat ; NBD:nucleotide-binding domain
PYD:pyrindomain; CARD:caspaseactivating and recruitment domain

### a) Familial cold autoinflammatory syndrome (FCAS)

FCAS, also known as familial cold urticaria, is at the benign end of the CAPS spectrum, and has the most favorable prognosis of all the cryopyrinopathies [32, 35]. FCAS is characterized by episodes of low-grade fever, polyarthralgia, and nonpruritic pseudourticarial rash appearing 1-2 hours after cold exposure (range: 30 min to 6 h) and persisting for approximately 12 hours [37, 36]. Other commonly reported symptoms include conjunctivitis, profuse sweating, dizziness, headache, nausea, and extreme thirst. Symptoms are most intense in young adults, but may begin as early as childhood. Less commonly, the syndrome may present as recurrent fever, mild arthralgia, inflammatory cardiomyopathy, nephropathy, and thyroiditis, with no skin involvement. Secondary amyloidosis is





the main cause of death, occurring in up to 2% of cases [37]. Treatment includes prevention of cold exposure and, in more severe cases, anakinra. A recent study of rilonacept, a long-acting soluble receptor that binds IL-1, found good efficacy and safety in 44 patients with FCAS. NSAIDs and corticosteroids are variably effective, and antihistamines are not effective at all [38].

**b) Muckle-Wells syndrome (MWS)**

In 1962, Muckle & Wells described a familial syndrome of urticaria, deafness, and amyloidosis affecting nine individuals [39]. The symptoms of MWS arise in childhood, as an urticaria-like rash with low-grade fever and arthralgia. Recurring episodes of arthritis and conjunctivitis may also occur. The most characteristic manifestation of MWS is sensorineural hearing loss, which is due to chronic inflammation of the organ of Corti with cochlear nerve atrophy [40]. Less common findings include oral and genital ulcers, cystinuria, ichthyosis, recurrent abdominal pain, and microscopic hematuria. Secondary amyloidosis is common, and may occur in 1/3 to 1/4 of patients. A finding of NLRP3 mutation confirms the diagnosis. Other laboratory findings include thrombocytopenia, anemia, and increased levels of acute-phase reactants [41]. As in the other cryopyrinopathies, IL-1 receptor inhibition with anakinra can reverse the clinical manifestations of MWS, including hearing loss.

**c) Neonatal onset multisystem inflammatory disease/chronic infantile neurologic, cutaneous, and articular syndrome (NOMID or CINCA syndrome)**

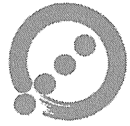
NOMID, or CINCA syndrome, is the most severe phenotype of the cryopyrinopathy spectrum, and was first described by Prieur & Griscelli in 1981 [42]. The disease is characterized by a triad of rash, chronic aseptic meningitis, and arthropathy. Clinical manifestations arise in the first weeks of life; the cutaneous lesions often appear within hours of birth [43]. Inflammatory symptoms (such as fever) are practically continuous, with occasional flares, and affected children have severe growth retardation.

Skin lesions are found in nearly 100% of cases. CNS involvement is the second most common feature, typically presenting as chronic aseptic meningitis with leukocyte infiltration of the cerebrospinal fluid, which leads to a broad range of symptoms including chronic irritability, headaches, seizures, transient hemiplegia, and lower limb spasticity. If left untreated, approximately 80% of patients will develop sensorineural hearing loss and ocular disease, such as conjunctivitis, anterior and posterior uveitis, papilledema, and optic nerve atrophy with loss of vision [44]. Other findings include developmental delay and mental retardation. Patients with NOMID/CINCA syndrome have a typical facial appearance, characterized by frontal bossing, macrocrania, and saddle nose. The musculoskeletal changes of NOMID/CINCA syndrome can range from asymptomatic arthritis to deforming arthropathy. Most patients show inflammatory changes of the long-bone epiphyses and metaphyses, with abnormal epiphyseal calcification and cartilage overgrowth, leading to shortened limbs and joint deformities. Premature ossification of the patella, with symmetrical patellar overgrowth, is a characteristic finding [43]. The typical arthropathy of NOMID is found in 50% of patients [43].

Nonspecific laboratory changes are as in other autoinflammatory syndromes, and may include anemia, thrombocytosis, moderately increased white blood cell counts, and increased inflammatory markers, such as ESR and CRP levels. The diagnosis of NOMID/CINCA syndrome relies on adequate clinical suspicion and confirmatory genetic testing. However, only 50% of patients with a characteristic presentation of NOMID/CINCA syndrome have NLRP3 mutations, which suggests that other yet-unknown genes may also be involved in its pathophysiology.

Without early identification and treatment, the prognosis for patients with NOMID/CINCA syndrome is guarded. In addition to deforming articular involvement and neurologic sequelae, the disease carries a high risk of secondary amyloidosis in the few patients who live to adulthood. Anakinra, an IL-1 receptor antagonist, is currently the drug of choice for treatment of NOMID and has been widely used in this indication, providing significant improvement in all clinical manifestations of the disease and, consequently, patient quality of life [43]. Corticosteroids and NSAIDs can provide symptomatic relief, but have no effect on articular or neurologic involvement.

Recently, canakinumab, which targets selectively human IL-1ß with high affinity and prevents the cytokine from interaction to its receptor, is reported to effectively block the inflammatory response in CAPS. In all studies performed, canakinumab



showed a rapid improvement of symptoms of CAPS and a complete clinical response was achieved in most patients. Inflammatory markers such as C-reactive protein and serum amyloid-A protein were reduced to normal levels within few days. In comparison to other IL-1 blockers, canakinumab provides a longer plasma half-life and less injection site reactions [45, 46].

## Mevalonate kinase deficiency (MKD) or hyper-IgD and periodic fever syndrome (HIDS)

HIDS follows an autosomal recessive pattern of inheritance, and is most often diagnosed in Northeastern Europe. The disease is caused by mutations in the MVK (mevalonate kinase) gene, which was discovered in 1999 [47]. MVK, which has 11 exons and is located on the long arm of chromosome 12 (locus 12q24), codes for mevalonate kinase (MK), a 396-amino acid-long enzyme. Most patients have a combination of two mutations, one of which is very often V377I. HIDS-associated mutations lead to a major reduction in MK activity (1 to 10% of normal levels), whereas mutations that completely eliminate MK function lead to a condition known as mevalonic aciduria (MA) [48, 49]. MA is a rare disease characterized by periodic fever with severe CNS involvement, mental retardation, ataxia, myopathy, poor growth, and early death.

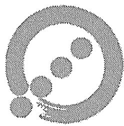
MK plays an essential role in the isoprenoid and cholesterol synthesis pathways. It catalyzes the conversion of mevalonic acid to mevalonate 5-phosphate during the synthesis of molecules such as cholesterol, vitamin D, biliary salts, corticosteroids, and non-steroidal isoprenoid compounds. During cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (the enzyme inhibited by statins) converts HMG-CoA to mevalonate, which is then phosphorylated to mevalonate phosphate. Mutations in the MVK gene block this pathway, preventing the conversion of mevalonate to mevalonate phosphate. The absence of a negative feedback loop, which is naturally provided by the presence of the end products of synthesis, leads to increased HMG-CoA reductase activity, consequently increasing serum, tissue, and urine levels of mevalonic acid. *In vitro* studies have shown that reduced synthesis of isoprenoids is associated with increased production of IL-1β [50]. Another recent in vitro study showed that MK inhibition leads to increased secretion of IL-1β due to activation of caspase-1, the enzyme that catalyzes formation of active IL-1β from its precursor [51]. High levels of immunoglobulin D (IgD) are characteristic of HIDS, but are apparently not associated with the severity of pathophysiology of the condition [52].

In MKD, febrile attacks occur more frequently in the first year of life, lasting 3 to 7 days and recurring every 4 to 6 weeks. However, the time elapsed between episodes can vary from patient to patient and even in a single individual. Febrile episodes recur for years, most frequently in childhood and adolescence, but months to years can go by between flares. Episodes may be triggered by immunization, trauma, surgery, or stress, and are characterized by high fever preceded by chills. Lymphadenopathy is extremely common. It is usually cervical, bilateral, and painful. Abdominal pain is also a frequent symptom, and may be accompanied by vomiting and/or diarrhea. Patients will also frequently report headache, and splenomegaly and hepatomegaly are common. Polyarthralgia and non-erosive arthritis of the large joints, particularly of the knees and ankles are also common. Arthritis is usually polyarticular and symmetric. Most patients have diffuse cutaneous lesions, which may consist of erythematous maculopapular rash, urticaria-like rash, erythematous nodules, petechiae, or purpura. Febrile episodes may be accompanied by sudden increase in acute phase reactant levels, including neutrophilic leukocytosis and elevated ESR, CRP, and SAA. Measurement of urinary mevalonate levels during attacks may be useful, particularly in patients with normal IgD levels.

IgD levels are persistently high ($\geq$100 U/mL) in most patients. Nonetheless, IgD levels may be within normal limits in some HIDS patients, especially children under the age of 3 [52]. Furthermore, the finding of high IgD levels is not specific for HIDS, as it occurs in other inflammatory diseases, such as FMF and TRAPS.

The diagnosis of MKD is confirmed by a finding of MVK mutations. However, the presence of a clinical phenotype consistent with the disease in conjunction with high serum IgD and urinary mevalonate levels may suggest the diagnosis.

Most of the usual treatments, such as NSAIDs, corticosteroids, IVIG, colchicine, and thalidomide, are ineffective in HIDS. The involvement of MK in the cholesterol synthesis pathway has encouraged the introduction of statins in the management of MKD; the efficacy of simvastatin, an HMG-CoA reductase inhibitor, has been demonstrated in 5/6 of MKD pa-





tients [53]. Use of etanercept and anakinra in refractory cases has also been reported [54, 55, 56]. Recently two patients with MVA have been treated successfully with stem cell transplantation [57].

## Pyogenic aseptic arthritis, pyoderma gangrenosum, and acne syndrome (PAPA syndrome)

PAPA syndrome is an autosomal dominant disease characterized by sterile, deforming arthritis, skin ulcers (pyoderma gangrenosum), and severe cystic acne. Unlike other autoinflammatory syndromes, PAPA does not have fever as its most prominent symptom.

PAPA syndrome is caused by mutations in the gene that codes proline-serine-threonine phosphatase interacting protein 1 (PSTPIP1), and only five associated mutations have been reported thus far. PSTPIP1 is a 416-amino acid-long protein expressed mostly in neutrophils. Mutations in PSTPIP1 are believed to lead to hyperphosphorylation of the protein, which could increase the potency of its binding to pyrin, with subsequent activation of IL-1β production, as seen in FMF [58].

## Deficiency of interleukin-1–receptor antagonist (DIRA)

A new autosomal recessive AIS, caused by mutations in the IL1RN gene, which codes for interleukin-1 receptor antagonist (IL1Ra), was reported recently [59]. The syndrome, which was described in 10 patients, was given the name “deficiency of interleukin-1 receptor antagonist” (DIRA) and is characterized by early onset of symptoms, most frequently in the neonatal period.

Patients with DIRA present with pustulosis, multifocal aseptic osteomyelitis, and markedly elevated ESR and CRP levels. Skin involvement may range from sparse pustules to generalized pustular dermatitis or ichthyosiform lesions. Skin biopsy may reveal neutrophilic infiltration of the epidermis and dermis, pustules in the stratum corneum, acanthosis, and hyperkeratosis. All patients described in the report had osteomyelitis, characterized by pain with movement and periarticular swelling; the most frequent radiological findings were widening of the costal arches, periosteal elevation along long bones, and multifocal osteolytic lesions.

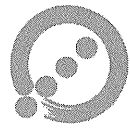
As in the other pyogenic autoinflammatory syndromes (PAPA and Majeed syndrome), fever is not a striking feature of DIRA, and was not present in any of the patients described. Two of the 10 patients had interstitial lung disease, and three died before therapy could be attempted (at 2 months, 21 months, and 9 years of age respectively).

The treatment of choice is recombinant IL-1RA (anakinra), which produces a dramatic response in skin and bone symptoms and in the quality of life of patients with DIRA.

## Pitfall for diagnosis of NOMID/CINCA syndrome

Recent genetic studies revealed that CAPS patients usually carry heterozygous mutations in the NLRP3 coding region (mutation positive patients) [60, 61, 62, 63, 64, 65]. Although they exhibit no recognizable differences in clinical symptoms or in their response to treatment, approximately half of CINCA syndrome patients lack detectable mutations in NLRP3, as assessed by conventional genomic sequencing (mutation-negative patients) [32, 60, 61, 62, 66, 67], indicating the existence of genetic heterogeneity among CAPS patients. Recently, we reported a patient with CINCA syndrome exhibiting mosaicism of a disease-associated mutation of NLRP3 [68]. This case suggested that some mutation-negative CAPS patients might have mosaicism of the NLRP3 mutation; however, the contribution of NLRP3 mosaicism to disease is controversial. Aksentijevich et al claimed that NLRP3 mosaicism is a rare event in mutation-negative patients, based on their analysis of 14 patients in which NLRP3 mosaicism was not identified, even with careful bidirectional sequencing [32, 68].

Somatic mosaicism has been reported in a number of autosomal dominant monogenic diseases [69]. Diagnosis of mosaicism by conventional genomic sequencing using the dideoxy termination method is often difficult, because the overlapping chromatogram of the mutant is easily missed when the frequency of a mutant allele is less than 20% to 30%. Heteroduplex-based methods or subcloning-based analysis of mutant alleles enable one to detect such low-level mosaicism; however, these methods are resource intensive, and cannot distinguish whether the detected mutation is disease-causing or simply a nonfunctional single nucleotide polymorphism (SNP). An alternative approach involves the isolation of mutant cells using functional analyses based on their characteristic biologic features, and then determining the DNA sequence of the isolated cells. Based on these backgrounds, we set out to identify specific biologic features of NLRP3-mutant cells compared



with nonmutated cells, in an effort to specifically isolate NLRP3-mutated cells from mutation-negative patients [70].

Disease-associated NLRP3 mutations induce ASC-dependent NF-κB activation in some systems, and we reported that they also induce necrotic cell death in the human monocytic cell line THP-1, which is a novel function of NLRP3 [68]. Based on these backgrounds, we explored whether NLRP3-mutant cells have specific biologic features, using monocytes from mutation-positive patients, and found that NLRP3-mutant monocytes rapidly underwent necrosis-like cell death after treatment with lipopolysaccharide (LPS) to induce NLRP3 expression. This unique phenotype of NLRP3 mutant cells enabled us to differentiate NLRP3-mutated cells and nonmutated cells in 3 of 4 mutation-negative CAPS patients, and we were able to successfully demonstrate that these 3 patients had mutations of NLRP3 as latent mosaicism (Fig. 3) [71, 72].

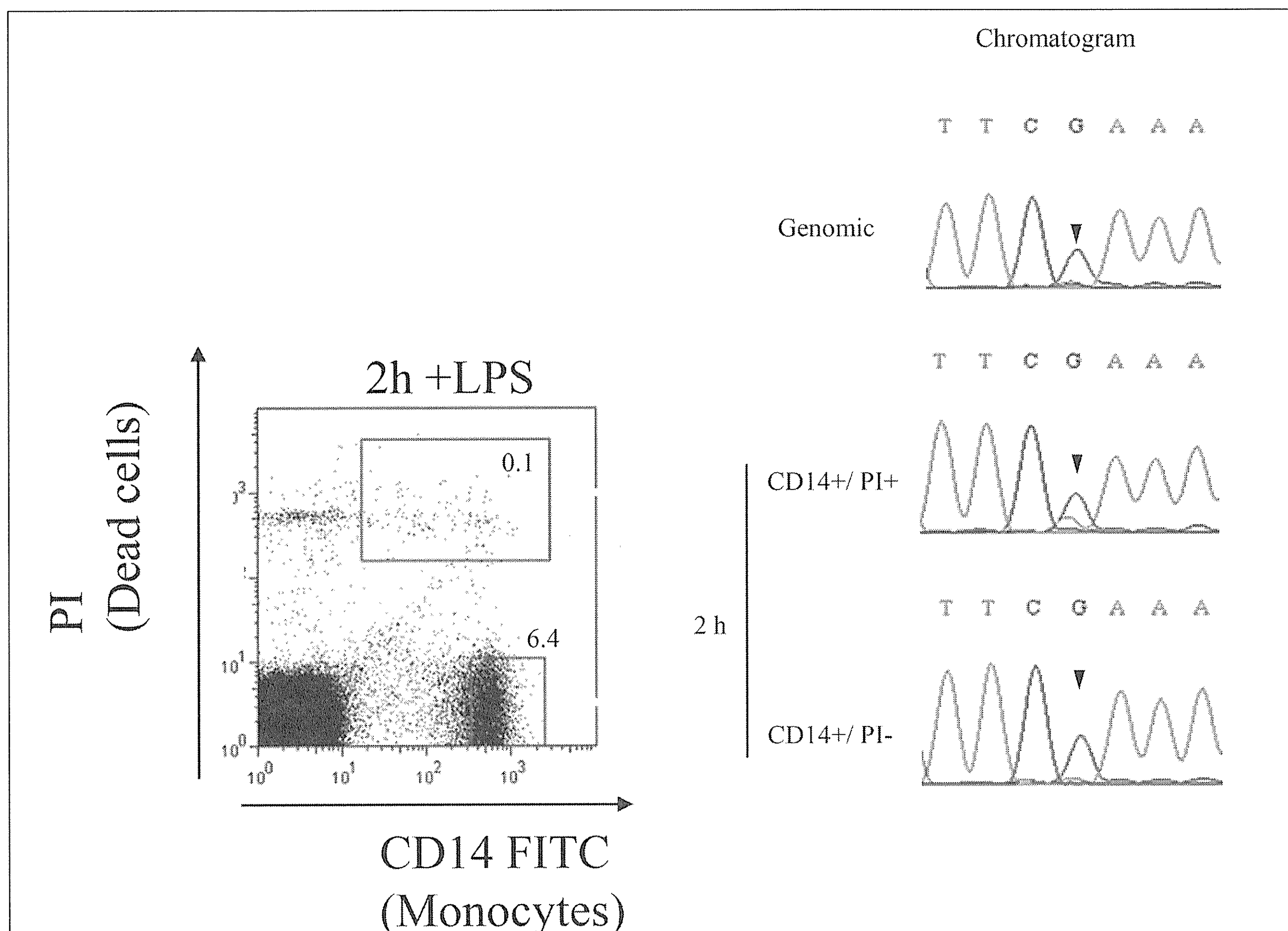



**Fig. 3 Enrichment of dying monocytesin mutation-negative patient revealed latent overlapping peaks**

PBMCs from mutation-negative CAPS patient were cultured with or without LPS (10 ng/mL) for 24 hours. CD14-positive and -negative cells were sorted, and DNA was extracted and sequenced for analysis of NLRP3.

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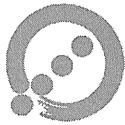



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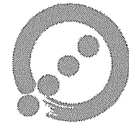

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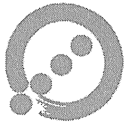



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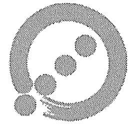

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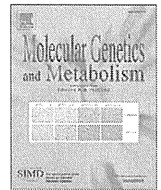

# Generation of induced pluripotent stem (iPS) cells derived from a murine model of Pompe disease and differentiation of Pompe-iPS cells into skeletal muscle cells

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ABSTRACT

Our study is the first to demonstrate the ability to generate iPS cells from a mouse model of Pompe disease. Initially, mouse tail tip fibroblasts were harvested from male, 8-week-old (GAA) knockout mice, and three reprogramming factors (Oct3/4, Sox2 and Klf4) were transfected into the isolated donor cells using a retroviral vector. These iPS cells also showed decreased levels of GAA enzymatic activity and strong positive staining with periodic acid-Schiff (indicating the accumulation of glycogen) and acid phosphatase (lysosomal activation marker). Pompe-iPS cells were differentiated into skeletal muscle cells in Matrigel®-coated plates. Spindle-shaped skeletal muscle cells were successfully generated from Pompe-iPS cells and showed spontaneous contraction and positive staining with the myosin heavy chain antibody. Electron microscopic analysis of the skeletal muscle cells showed typical morphological features, including Z-bands, I-bands, A-bands and H-bands, which were visible in wild-type and Pompe cells. Furthermore, Pompe skeletal muscle cells accumulated massive glycogen in lysosomes. This study indicates that the iPS and skeletal muscle cells generated in this study could also be a useful disease model for studies investigating the pathogenesis and treatment of skeletal muscle in Pompe disease.

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## 1. Introduction

Pompe disease (GSD-II; glycogen storage disease type II) accumulates glycogen in various cells throughout the body due to a deficiency of acid-α-glucosidase (GAA) [1]. Clinically, there are two types of Pompe disease: infantile and late onset. The main clinical symptoms of infantile Pompe disease are cardiac hypertrophy with congestive heart failure, muscle weakness and respiratory failure, whereas the late onset type is characterized by muscle weakness, gait disturbances and respiratory failure [2,3]. Enzyme replacement therapy (ERT) is available for Pompe disease. Treatment for the infantile form has been shown to improve survival and motor outcomes compared to untreated controls [4–6]. Clinical trial data for the treatment of children and adult patients with Pompe disease has shown some positive effects associated with improved walking distance, stabilization of pulmonary function and improved QOL [7–9]. However, the treatment of patients with late-stage disease remains difficult [10,11]. Induced pluripotent stem (iPS) cell technology may facilitate the study of the pathogenesis of Pompe disease caused by different mutations in the GAA gene and might eventually enable the development of autologous cell transplantation therapy without immunological problems.

We, therefore, generated iPS cells from somatic cells (e.g., fibroblasts and lymphocytes) by introducing reprogramming factors (Oct3/4, Sox2, Klf4 and c-Myc) according to the method described by Takahashi et al. [12].

In this study, we generated iPS cells from the fibroblasts of a murine model of Pompe disease and also differentiated iPS cells into skeletal muscle cells.

*Abbreviations:* GAA, Acid α-glucosidase; ERT, Enzyme replacement therapy; iPS, Induced pluripotent stem; Wt-iPS, Wild type-iPS; EB, Embryoid body.

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The iPS technology may facilitate the study of the pathogenesis of Pompe disease caused by different mutations in the GAA gene and might eventually enable the development of autologous cell transplantation therapy without immunological problems. The method for deriving the skeletal muscle lineage cells from ES and iPS cells in vitro was established by Chang et al. and Mizuno et al. [13,14]. In this study, we generated iPS cells from a mouse model of Pompe disease and also succeeded differentiated Pompe iPS cells into skeletal muscle which showed massive accumulation of glycogen in lysosomes.

## 2. Materials and methods

### 2.1. Mice

GAA-knockout mice were provided by Dr N Raben (NIH, Bethesda, MD, USA). Nude mice (BALB/cAJcl-nu/nu) were purchased from CLEA (Tokyo, Japan).

All animal experiments were approved by the Jikei University's Animal Experiments Committee.

### 2.2. Cell culture and generation of iPS cells

Pompe-iPS cells were generated from the fibroblasts of a GAA knockout mouse (aged 8 week, male) using three reprogramming factors (Oct3/4, Klf4, Sox2) according to the protocol described by Takahashi et al. [12].

Three-factor wild-type-iPS (Wt-iPS) cells, which lack c-Myc (iPS- MEF-Ng-178B-5), were purchased from the Riken cell bank established by Nakagawa, et al. [15].

### 2.3. RT-PCR

Total RNA was extracted from cultured iPS cells using RNeasy (QIAGEN, Hilden, Germany) according to the protocol described by Meng et al. [16] with primers and PCR conditions described previously [17].

### 2.4. Immunostaining and cytochemical staining

The immunostaining study was performed as described previously [18].

The primary antibodies used were SSEA-1 (480; CD15; Santa Cruz biotechnology, CA, USA), actinin (Sarcomeric; clone EA-53; Sigma-Aldrich, MO, USA), alpha-fetoprotein (clone 189502; R&D Systems, MN, USA Inc.), beta-tublin III (TUJ1; Babco,NJ, USA) and myosin (clone MY- 32; Abcam, Cambridge shire, UK). Cytochemical staining was performed using alkaline phosphatase (Sigma-Aldrich), PAS and acid phosphatase staining kits (#15792 & #15732; MUTO PURE CHEMICALS, Tokyo, Japan) according to the manufacturers' recommendations.

### 2.5. Teratoma formation

The teratoma formation study was performed as previously described by Meng et al. [16]. Then, the iPS cells that eliminated the feeder cells were suspended at $1 \times 10^6$ cells/100 µl in DMEM supplemented with 10% fetal bovine serum (FBS) and injected subcutaneously into the dorsal flank of 4-week-old female nude mice. After 4 weeks, the tumors were dissected and fixed with a 10% formaldehyde neutral buffer solution (WAKO), embedded in paraffin, and the sliced sections were stained with hematoxylin and eosin.

### 2.6. Enzyme assay

The iPS cells that eliminated the feeder cells were homogenized in sterile water. The GAA activity of the iPS cells was determined with the fluorogenic substrate 4-methylumbelliferyl α-D-glucopyranoside (Sigma-Aldrich) as previously described [19].

### 2.7. Differentiation of the iPS cells into skeletal muscle cells in vitro

Differentiation of the iPS cells was based on a previously established method [13,14]. Therefore, after the removal of the feeder cells, the iPS cells treated with 0.25% trypsin/EDTA (Sigma-Aldrich), were plated onto tissue culture dishes coated with 0.1% gelatin (Sigma-Aldrich) and incubated for 1 h. Then, the supernatant was collected.

For embryoid body (EB) formation, murine iPS cells removed from the feeder cells were transferred to a 96-well ultra low attachment culture plate (CORNING, MA, USA) at a concentration of 800 cells/well in differentiation medium consisting of DMEM (WAKO, Osaka, Japan) supplemented with 10% FBS (GIBCO, CA, USA), 5% horse serum (GIBCO), 0.1 mM 2-mercaptoethanol (GIBCO), 0.1 mM nonessential amino acids (GIBCO), and 50 µg/ml penicillin/streptomycin (GIBCO). After 3 days, the EBs were transferred to differentiation medium, and the EBs were attached to a 48-well tissue culture plate (NUNC, MA, USA) coated 50 µl Matrigel® Basement Membrane Matrix (BD Bioscience, Belford MA, USA) after 6 days.

### 2.8. Electron microscopy

The iPS cells were doubly fixed with 2% glutaraldehyde/0.1 M phosphate buffer (PB) (pH 7.2) and 1% osmium tetroxide/0.1 M PB (pH 7.2) and were dehydrated with an ethanol gradient. Then, the cells were embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a Hitachi H7500 electron microscope (Hitachi, Tokyo).

## 3. Results

### 3.1. Analysis of generated Pompe-iPS cells for the undifferentiated state and pluripotency

We generated iPS cells from mouse tail tip fibroblasts (Fig. 1A) from a GAA knockout mouse (male, 8 weeks old) using established reprogramming factors, Oct3/4, Klf4, and Sox2. Then, iPS colonies were confirmed 28 days after transfection (Fig. 1B).

These colonies showed bright cytoplasm and a rounded configuration on the SNL feeder cells. To confirm the undifferentiated state, we performed RT-PCR using 13 ES cell marker genes that are expressed in putative iPS cells (Fig. 1C). Clone no. 7 expressed all of the ES marker genes. The isolated iPS cells had high alkaline phosphatase activity (AP; Fig. 1D) and were strongly positive for stage-specific embryonic antigen 1 (SSEA-1; Fig. 1E). These results demonstrated that the isolated putative iPS clone could be reprogrammed into a pluripotent cell. We evaluated the pluripotency of the generated Pompe-iPS cells using a teratoma formation study. An 8-mm teratoma was formed in a nude mouse (Fig. 1F) 4 weeks after an injection of the iPS clone. A histological examination revealed that the teratoma could differentiate into three germ layers, including α- smooth muscle actin (mesoderm marker), α-fetoprotein (endoderm marker), and β-tubulin III (ectoderm marker) (Figs. 1G–I). Thus, the generated Pompe-iPS cells were confirmed to be pluripotent for three germ layers.

### 3.2. Characterization of generated Pompe-iPS cells for the disease phenotype

Isolated Pompe-iPS cells demonstrated significantly decreased levels of GAA activity for the Pompe disease phenotype compared to the Wt-iPS cells (Fig. 2A).

**Fig. 1.** Analysis of generated Pompe-iPS cells from mouse model of Pompe disease. (A) Morphology of tail tip fibroblast for model mouse of Pompe disease. (B) Generated Pompe-iPS cells on SNL feeder cells after 28 days from transfection. (C) Analysis of RT-PCR for isolated Pompe-iPS cells using ES marker gene. Nat1 is internal control. ttf (tail tip fibroblast from model mouse of Pompe-iPS cells) and snl (SNL feeder cells) are negative controls, and ES (ES cells) is positive control. (D,E) isolated iPScells colone no.7 was positive for alkaline phosphatase and SSEA-1. Black bar = 500 μm. Red bar = 412.5 μm. (F–I) The pluripotency of Pompe-iPS cells was evaluated by teratoma formation. Teratoma was formed into nude mouse, approximately 8 mm in diameter (black arrow). Immunostaining on teratoma was positive for α-smooth muscle actin (G), α-fetoprotein (H), and β-tubulin III (I).

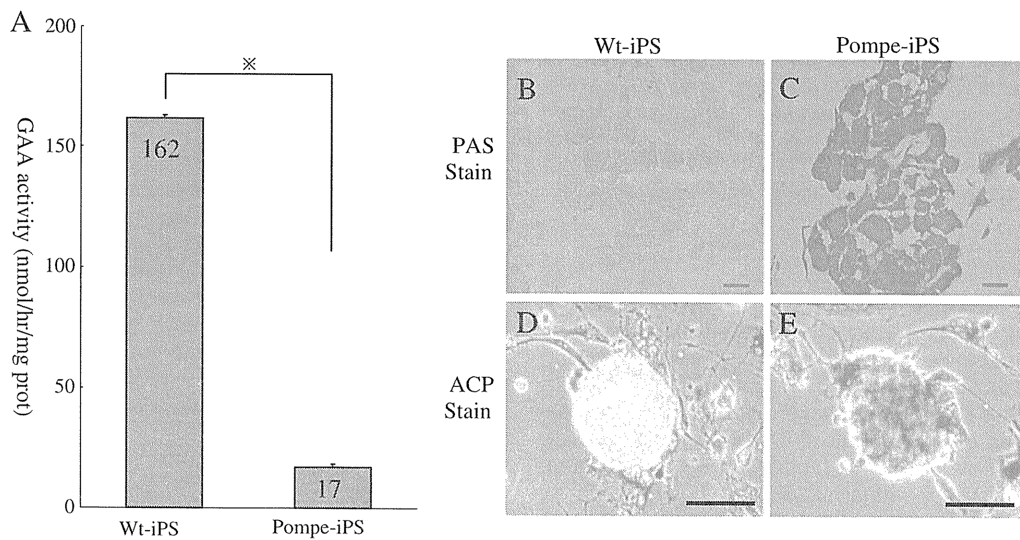


**Fig. 2.** Generated Pompe-iPS cells showed a typical Pompe disease phenotype. (A) Pompe-iPS cells were confirmed by low GAA enzyme activity compared (n = 3). Wt-iPS = 162 ± 1.27 nmol/hr/mg protein, Pompe-iPS = 17 ± 1.26 nmol/h/mg protein ( P<0.005). (B, D) Wt-iPS cells showed less staining for PAS stain and ACP stain. (C, E) Pompe-iPS cells showed strong positive staining for PAS stain and ACP stain. Red bar = 10 μm. Black bar = 100 μm.

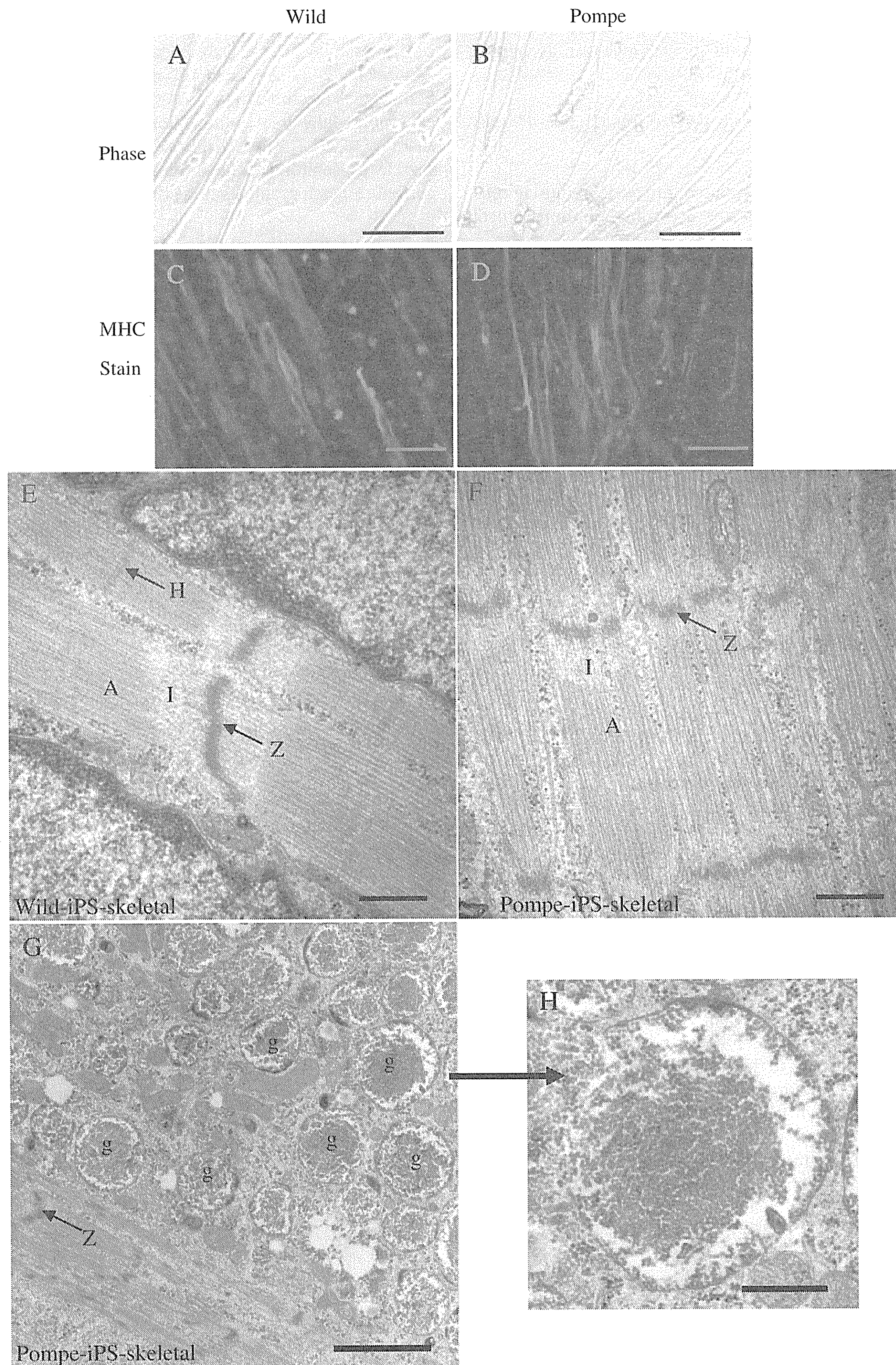


**Fig. 3.** Morphological features of skeletal muscle lineage cells from Wt and Pompe-iPS cells.(A, B) Skeletal muscle lineage cells from Wt and Pompe-iPS cells (B). black bar = 200 μm. (C, D) These cells were strongly positive for anti-MHC. Blue bar = 165 μm. (E, F) We observed H-band (H), A-band (A), I-band (I), and Z-band (Z) from spindle-shaped fiber cells on day 41. Direct mag = 40,000×, bar = 500 nm. (G) Furthermore, we confirmed accumulation of glycogen (g) into Pompe-iPS skeletal cells. direct mag = 12,000×, bar = 2 μm. (H) High magnification of figure (G) shows a typical glycogen accumulation surrounded by a unit membrane. Direct mag = 25,000×, bar = 500 nm.