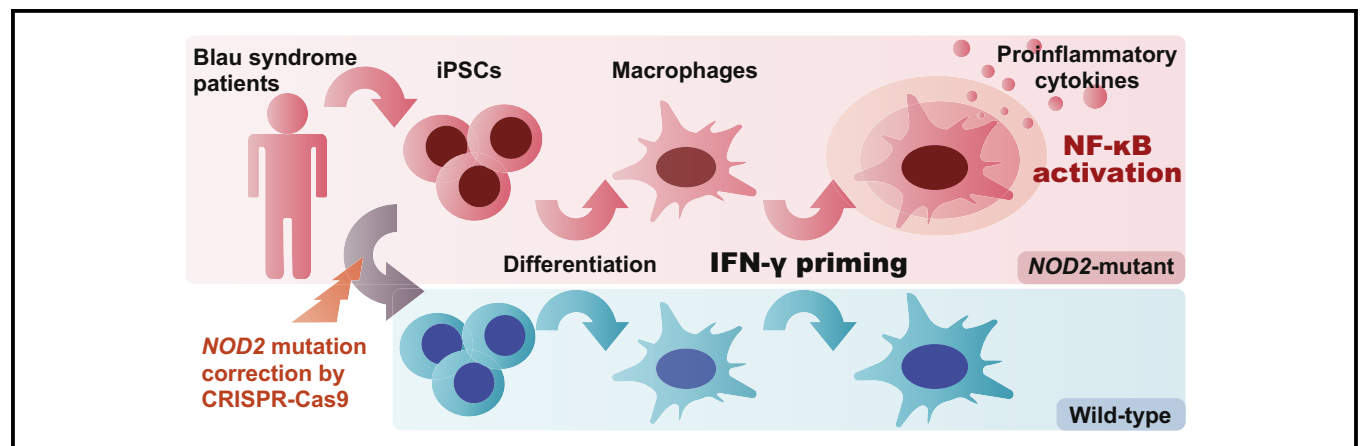


Pluripotent stem cell models of Blau syndrome reveal an IFN- γ -dependent inflammatory response in macrophages

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GRAPHICAL ABSTRACT



Background: Blau syndrome, or early-onset sarcoidosis, is a juvenile-onset systemic granulomatosis associated with a mutation in nucleotide-binding oligomerization domain 2 (*NOD2*). The underlying mechanisms of Blau syndrome leading to autoinflammation are still unclear, and there is currently no effective specific treatment for Blau syndrome.

Objectives: To elucidate the mechanisms of autoinflammation in patients with Blau syndrome, we sought to clarify the relation between disease-associated mutant *NOD2* and the inflammatory response in human samples.

Methods: Blau syndrome-specific induced pluripotent stem cell (iPSC) lines were established. The disease-associated *NOD2*

mutation of iPSCs was corrected by using a CRISPR-Cas9 system to precisely evaluate the *in vitro* phenotype of iPSC-derived cells. We also introduced the same *NOD2* mutation into a control iPSC line. These isogenic iPSCs were then differentiated into monocytic cell lineages, and the statuses of nuclear factor κ B pathway and proinflammatory cytokine secretion were investigated.

Results: IFN- γ acted as a priming signal through upregulation of *NOD2*. In iPSC-derived macrophages with mutant *NOD2*, IFN- γ treatment induced ligand-independent nuclear factor κ B activation and proinflammatory cytokine production. RNA sequencing analysis revealed distinct transcriptional profiles of

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mutant macrophages both before and after IFN- γ treatment. Patient-derived macrophages demonstrated a similar IFN- γ -dependent inflammatory response.

Conclusions: Our data support the significance of ligand-independent autoinflammation in the pathophysiology of Blau syndrome. Our comprehensive isogenic disease-specific iPSC panel provides a useful platform for probing therapeutic and diagnostic clues for the treatment of patients with Blau syndrome. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

Key words: Blau syndrome, nucleotide-binding oligomerization domain 2, nuclear factor κ B, IFN- γ , disease-specific induced pluripotent stem cells

Blau syndrome (OMIM: #186580), or early-onset sarcoidosis, is a hereditary juvenile-onset systemic granulomatosis.¹ Clinical symptoms appear before the age of 4 years and mainly affect the skin, joints, and eyes.² The symptoms are progressive and cause severe complications, such as joint destruction and blindness.³ The pathologic findings of affected lesions include granuloma formation mainly composed of macrophages with other cells, such as multinuclear giant cells and lymphocytes,⁴ which is associated with local chronic inflammation. Although TNF- α antagonists are partially effective for controlling some symptoms, such as arthritis,^{5,6} there is no specific curative treatment at present for patients with Blau syndrome.⁷

Heterozygous mutations of nucleotide-binding oligomerization domain 2 (*NOD2*) or caspase recruitment domain-containing protein 15 (*CARD15*) were identified as responsible for the onset of Blau syndrome.⁸ *NOD2* protein is an intracellular pathogen recognition receptor, the ligand of which is known as muramyl dipeptide (MDP), found in bacterial cell walls.^{9,10} On recognition of the ligand, *NOD2* leads to activation of the nuclear factor κ B (NF- κ B) pathway, thereby causing upregulation of proinflammatory cytokines and chemokines.⁹⁻¹³ However, the details regarding the molecular mechanisms by which Blau syndrome-associated mutant *NOD2* leads to autoinflammation and granuloma formation are still unclear.

Because of several controversial reports, whether Blau syndrome-associated *NOD2* mutations are gain-of-function or loss-of-function mutations is still argued. For example, in a model of HEK293 cells, ligand-independent autoactivation of NF- κ B reporter activity was observed on overexpression of Blau syndrome-associated mutant *NOD2*, indicating that Blau syndrome is caused by gain-of-function mutations of *NOD2*.¹⁴ Conversely, a knock-in mouse model described a reduction in levels of proinflammatory cytokines responsive to the NF- κ B pathway on stimulation with MDP, showing the possibility of loss of function.¹⁵ However, because the mouse model did not show any phenotype of Blau syndrome *in vivo*, we recognized the need to investigate specific perturbations that abstract the unique function of mutant *NOD2* using human samples.

Although *NOD2* is mainly expressed in hematopoietic lineage cells, especially in monocytic cells,¹³ excessive production of proinflammatory cytokines or chemokines was not observed in PBMCs of patients with Blau syndrome.^{16,17} Although other patients with autoinflammatory syndrome usually experience periodic proinflammatory attacks, the clinical course of Blau syndrome is usually slow and progressive, without specific episodes of febrile attacks, which hampers evaluation of the disease activity of Blau syndrome. A lack of clinical parameters

Abbreviations used

ChIP:	Chromatin immunoprecipitation
DAPI:	4'-6-Diamidino-2-phenylindole dihydrochloride
gRNA:	Guide RNA
GSEA:	Gene set enrichment analysis
iPSC:	Induced pluripotent stem cell
iPS-ML:	Immortalized proliferating myeloid cell line
iPS-MP:	Immortalized proliferating myeloid cell line-derived macrophages
M-CSF:	Macrophage colony-stimulating factor
MDP:	Muramyl dipeptide
Mo-MP:	Monocyte-derived macrophages
NF- κ B:	Nuclear factor κ B
NOD2:	Nucleotide-binding oligomerization domain 2
qPCR:	Quantitative PCR
RNA-seq:	RNA sequencing
RUV:	Remove unwanted variation
SCF:	Stem cell factor
TPO:	Thyropoietin

associated with disease activity makes it difficult to elucidate the mechanisms of this disease.

Substantial evidence supports a potential role of IFN- γ in the pathophysiology of Blau syndrome. Prominent IFN- γ expression was found in and around the granulomas of biopsy specimens from patients with Blau syndrome, indicating its commitment in the progression of granuloma.⁴ IFN- γ is also described as a downstream mediator of *NOD2*-driven inflammation leading to uveitis.¹⁸ In some patients, BCG vaccination was sometimes associated with the onset of this disease,^{19,20} showing the possibility that a BCG-associated immune response triggers a *NOD2*-dependent inflammatory pathway, which leads to flare-up of Blau syndrome symptoms. Because IFN- γ is a major cytokine associated with BCG-mediated immune responses,²¹ it might be involved in provoking a *NOD2*-mediate inflammatory response. Furthermore, IFN- γ , TNF- α , and other Toll-like receptor ligands act as priming signals to upregulate *NOD2* expression.²²⁻²⁴ However, currently, there are no reports describing the direct effect of IFN- γ on Blau syndrome-associated immune cells.

Here we established that human induced pluripotent stem cell (iPSC) lines specific to patients with Blau syndrome have *in vitro* disease models for elucidating the mechanisms of autoinflammation in Blau syndrome.²⁵ The iPSC model is free from the influence of the *in vivo* cellular environment, such as medication or endogenous cytokines. Taking advantage of the nature of human pluripotent stem cells, in that their genome sequence can be edited relatively easily by using the CRISPR-Cas9 genome editing system,^{26,27} we established isogenic iPSC pairs sharing the same genomic backgrounds but different *NOD2* genotypes. In the present study we corrected the *NOD2* R334W mutation, which is one of the most common variants of Blau syndrome,²⁸ in iPSCs derived from patients with Blau syndrome. In a similar manner we introduced the *NOD2* R334W mutation into an iPSC clone from a healthy donor. Using macrophages derived from these isogenic iPSC panels, we investigated the relationship between Blau syndrome-associated mutant *NOD2* and the proinflammatory response.

METHODS

Ethical matters

This study was approved by the Ethics Committee of Kyoto University, and informed consent was obtained from each patient's guardians in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients. The PBMCs for iPSC generation were obtained from 2 independent patients with Blau syndrome (Blau1, CIRA00232; Blau2, CIRA00233).

Patients

Two Japanese boys with the *NOD2* R334W mutation given a diagnosis of Blau syndrome, as previously described,^{29,30} were recruited to establish iPSCs. The patients were brothers. Their father with the same *NOD2* mutation was also given a diagnosis of Blau syndrome, as previously described.^{14,20,30} Each of the 2 patients had similar clinical episodes showing that erythematous papules appeared after BCG vaccination at 6 months of age. Thereafter, one of the patients (Blau1) had a skin rash and arthritis without uveitis and received treatment with nonsteroidal anti-inflammatory drugs and methotrexate. Another patient (Blau2) had a skin rash without arthritis or uveitis and had not received any specific medication. PBMCs were obtained from another unrelated patient who had the same *NOD2* R334W mutation to obtain monocyte-derived macrophages (Mo-MPs).

Primers

The primers used in this study are listed in Table E1 in this article's Online Repository at www.jacionline.org.

Statistics

All of the statistical analyses were performed with the Excel (Microsoft, Redmond, Wash), R, and Prism (GraphPad Software, La Jolla, Calif) software programs. The values are presented as means $\pm$ SEMs or SDs, as indicated in the figure legends. Statistical analysis was done by using either the Student *t* test or the Wilcoxon rank sum test.

For more information, see the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS

Generation of iPSCs specific to patients with Blau syndrome and associated isogenic iPSC panels

We first established and characterized multiple iPSC lines from 2 patients with Blau syndrome with a heterozygous R334W (1000C>T) *NOD2* mutation (referred to as Blau1A-1F and Blau2A-2F, see Table E2 in this article's Online Repository at www.jacionline.org). iPSCs from patients with Blau syndrome were confirmed to carry the corresponding heterozygous point mutation on exon 4 of the *NOD2* gene, showed typical pluripotent stem cell-like morphology, maintained a normal karyotype, and expressed comparable levels of pluripotency-associated genes (*NANOG* and *OCT4*) to a control iPSC line (see Table E2). We selected representative iPSC clones from both patients (Blau1E and Blau2B) and named them Blau1-R334W (Blau1E) and Blau2-R334W (Blau2B). The data of these representative clones for the evaluation of iPSC quality are shown in Fig E1, A-C, in this article's Online Repository at www.jacionline.org. The pluripotency of iPSC clones from patients with Blau syndrome, including Blau1-R334W and Blau2-R334W, was confirmed by using a teratoma formation assay (see Fig E1, D, and Table E2).

To understand the pathophysiology derived from Blau syndrome-associated mutant *NOD2* in a strictly controlled isogenic

background, we next corrected the mutation in the Blau1-R334W and Blau2-R334W iPSC clones by using CRISPR-Cas9-mediated targeting (referred to as Blau1-corrected and Blau2-corrected, respectively; Fig 1, A).^{26,27} In the corrected clone (Blau1-corrected and Blau2-corrected) the inserted allele was validated by using genomic PCR (Fig 1, B, and see Fig E1, E), and the T to C correction of *NOD2* on exon 4 was confirmed by using Sanger sequencing (Fig 1, C, and see Fig E1, F). In a similar manner we also introduced a heterozygous *NOD2* R334W mutation into control 201B7-iPSCs (referred to as 201B7-WT for parental control 201B7 and 201B7-R334W for mutated 201B7; see Fig E1, G-I). To investigate the expression of endogenous *NOD2*, we generated 201B7-WT iPSCs with 3 $\times$ FLAG-tagged *NOD2* in the C-terminal region using the CRISPR-Cas9 system (referred to as 201B7-WT-NOD2-FLAG; see Fig E1, J and K).

Differentiation of iPSCs into a monocytic lineage

Because monocytic lineage cells, especially macrophages, express *NOD2*, produce inflammatory cytokines robustly, and play a pivotal role in granuloma formation, we first differentiated iPSCs into a monocytic lineage using a previously reported protocol.³¹ We tracked the expression of *NOD2* mRNA during differentiation and found the monocytic lineage cells derived from iPSCs expressed *NOD2* on day 12 of differentiation (Fig 1, D). The iPSC-derived monocytes were then further differentiated into macrophages and stimulated with IFN- γ or IL-4 to elicit polarization into M1 or M2 macrophages.³¹ Of note, the iPSC-derived macrophages treated with IFN- γ showed especially high expression of *NOD2*, as previously described (Fig 1, D).²²⁻²⁴ IFN- γ treatment upregulated the expression of *NOD2* protein (Fig 1, E), thus indicating a priming role of IFN- γ in the *NOD2*-NF- κ B signaling pathway.

Given these observations, we next investigated the *in vitro* function of *NOD2* in iPSC-derived macrophages. One of the obstacles facing iPSC disease studies is that directed differentiation from iPSCs is often time and labor intensive, and the functional analysis often shows high variance. To overcome these issues and stably obtain a scalable number of mature monocytic cells from engineered iPSC clones, immortalized proliferating myeloid cell lines (iPS-MLs) were established from iPSC-derived monocytes by introducing 3 transgenes: *cMYC*, *BM11*, and *MDM2*.³² The established iPS-MLs showed a monocytic appearance (see Fig E2, A, in this article's Online Repository at www.jacionline.org) and expressed CD33 but only expressed CD14 slightly (see Fig E2, B), indicating their characteristics of immature monoblast-like status. When the iPS-MLs were differentiated into macrophages (immortalized proliferating myeloid cell line-derived macrophages, [iPS-MPs]), expression of the macrophage cell-surface markers CD14, CD68, and CD11b were upregulated (see Fig E2, C). These iPS-MPs demonstrated an adherent cell appearance, and IFN- γ treatment gave them an M1 macrophage-like, elongated, tightly attached appearance (see Fig E2, D).

NF- κ B is similarly activated in iPS-MPs with wild-type and mutant *NOD2*

Activation of *NOD2* by its ligands, such as MDP, is involved in the immune response induced by NF- κ B activation.^{9,11,13} To

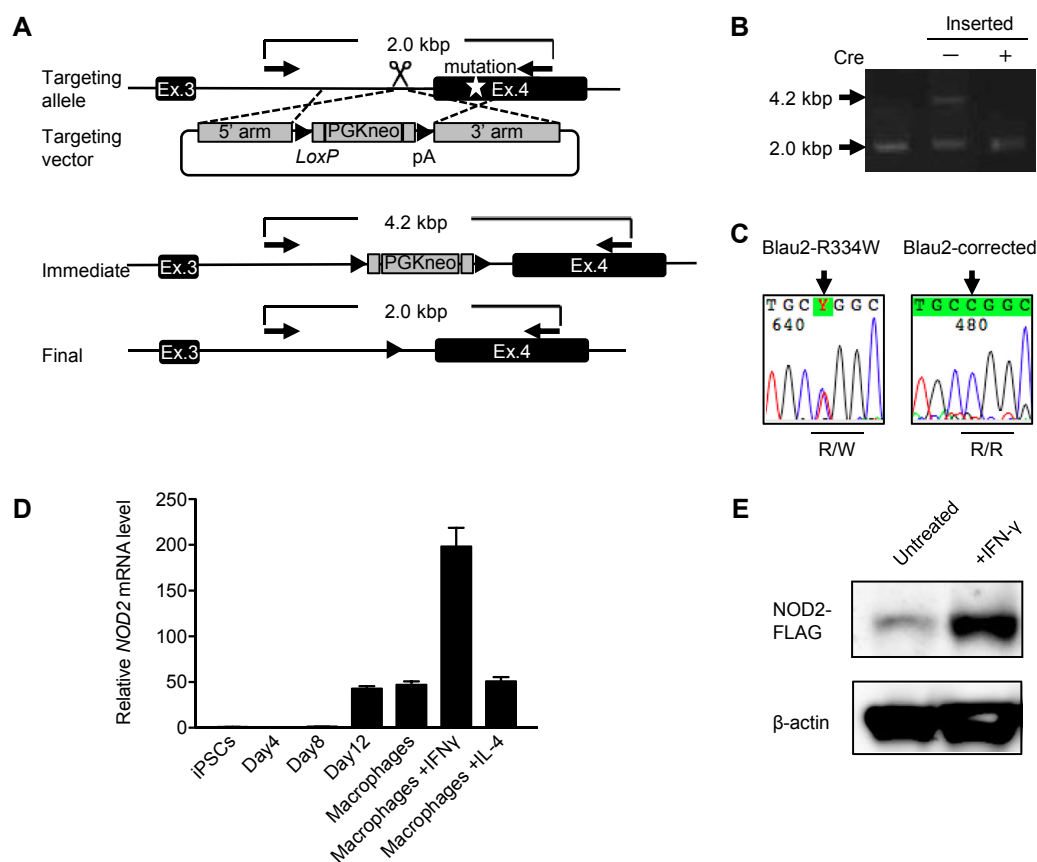


FIG 1. Establishment of an isogenic iPSC panel with Blau syndrome-specific clones. **A**, Schematic illustration of the stepwise gene correction of the *NOD2* R334W mutation by using the CRISPR-Cas9 system. Arrows indicate the PCR primers used in Fig 1, B, and Fig E1, E. **B**, Validation of the corrected allele by using genomic PCR. **C**, Representative chromatogram of Sanger sequencing of *NOD2*. **D**, Quantitative RT-PCR validation of *NOD2* expression during differentiation into macrophages derived from iPSCs. Data are presented as means $\pm$ SDs (n = 3). **E**, Western blotting of NOD2-FLAG protein in iPS-MPs treated with IFN- γ (50 ng/mL) for 24 hours. Data are from at least 3 independent experiments.

investigate activation of NF- κ B in cells with wild-type *NOD2* and *NOD2* mutations, we next tracked the nuclear translocation of NF- κ B p65, a subunit of the NF- κ B transcription complex, as a parameter of NF- κ B activation (Fig 2). In untreated iPS-MPs NF- κ B p65 localized mostly in the cytosol. On stimulation with MDP, NF- κ B p65 remained in the cytosol for a shorter time course (1-5 minutes after stimulation) and translocated into the nucleus after 30 minutes to 1 hour. With MDP treatment after 6 hours, NF- κ B p65 was returned into the cytosol. Overall, the response of NF- κ B to MDP stimulation was similar in cells with wild-type and mutant *NOD2*.

IFN- γ induces ligand-independent NF- κ B activation on *NOD2*-mutated iPS-MPs

To investigate the role of mutant *NOD2* on the immune response, we first investigated the NF- κ B transcription activity in iPS-MPs using a luciferase reporter system. Interestingly, IFN- γ priming significantly increased the luciferase activity in mutant iPS-MPs without secondary stimulation by MDP (Fig 3, A). The degree of NF- κ B activation in mutant iPS-MPs by IFN- γ increased in a dose- and time-dependent manner (see Fig E3, A and B, in this article's Online Repository at www.jacionline.org). Both

wild-type and mutant iPS-MPs were able to react to MDP at comparable levels of NF- κ B activation (Fig 3, A). The time course of the degree of NF- κ B activation in both the wild-type and mutant iPS-MPs by MDP peaked at 4 hours after stimulation and then started to decrease, which was earlier than observed with IFN- γ (see Fig E3, C). Activation of NF- κ B in cells treated with IFN- γ and MDP was greater than that in cells that were treated only with MDP in both wild-type and mutant iPS-MPs (Fig 3, A), suggesting a priming role of IFN- γ in the NOD2-NF- κ B signaling pathway. Activation of NF- κ B signaling with LPS, which is mainly mediated by the Toll-like receptor signaling pathway,³³ was not significantly different between wild-type and mutant clones (see Fig E3, A).

To evaluate the NF- κ B activation in each cell, we next investigated the nuclear translocation of NF- κ B p65. Some of the IFN- γ -primed cells in the mutant clone showed NF- κ B p65 nuclear translocation without MDP (Fig 3, B), suggesting a ligand-independent activation state. The immunofluorescent density of the nuclear translocated NF- κ B p65 in mutant iPS-MPs was significantly increased compared with that in wild-type cells (Fig 3, C). In contrast, untreated cells demonstrated no obvious spontaneous nuclear translocation of NF- κ B in either wild-type or mutant iPS-MPs (Fig 3, B and C). MDP treatment increased

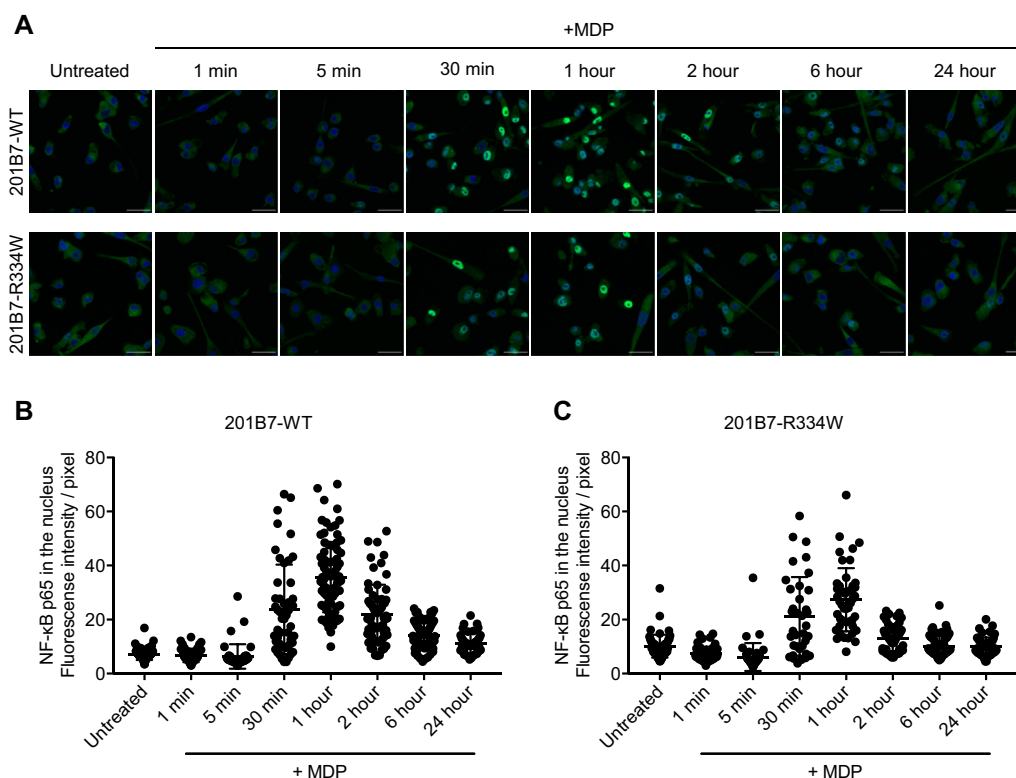


FIG 2. Time course of nuclear translocation of NF- κ B p65 in iPS-MPs by MDP. **A**, Representative confocal images of immunofluorescence staining of NF- κ B p65 (green). Cells were treated with MDP (10 μ g/mL) for the indicated times. Cells were stained with DAPI for visualization of the nucleus (blue). Scale bars = 40 μ m. **B** and **C**, Quantitation of nuclear translocation of NF- κ B p65 by means of immunofluorescence staining. Mean fluorescence intensity of NF- κ B p65 (green) merged with the nucleus (blue) in each cell was plotted. Bars indicate means $\pm$ SDs (n = 36-80). Data are from at least 3 independent experiments.

NF- κ B activity at comparable levels between wild-type and mutant iPS-MPs (Fig 3, B and C), as mentioned above in Fig 2.

Overall, IFN- γ priming induces ligand-independent activation of the NF- κ B pathway in mutant iPS-MPs, which might associate with an overproduction of proinflammatory cytokines.

IFN- γ induces proinflammatory cytokine production on *NOD2*-mutated iPS-MPs by increasing binding of NF- κ B p65 to their promoter regions

To evaluate the consequence of NF- κ B activation, we next measured the production of proinflammatory cytokines and chemokines (Fig 4, A-C). When untreated, production of proinflammatory cytokines from both wild-type and mutant clones was negligible. IFN- γ priming significantly increased the production of common NF- κ B-responsive cytokines, such as IL-6, IL-8, and TNF- α , in mutant iPS-MPs without secondary MDP stimulation, which is consistent with the status of the NF- κ B pathway. No marked differences in cell viability were noted with each stimulation, regardless of the genotype or type of stimulation (see Fig E4 in this article's Online Repository at www.jacionline.org), excluding the effect of cell death on cytokine profiles. On the other hand, despite comparable levels of NF- κ B activity, production of these cytokines with MDP in mutant iPS-MPs was significantly lower than that from wild-type iPS-MPs. No significant secretion of other cytokines, such as IL-1 β , IL-10, or IL-12p70,

was observed in either wild-type or mutant iPS-MPs under these conditions (see Fig E3, D-F).

A chromatin immunoprecipitation (ChIP) assay with NF- κ B p65 protein was performed to confirm that the increased cytokine production on IFN- γ -primed mutant iPS-MPs was associated with NF- κ B activation. IFN- γ priming induced ligand-independent recruitment of NF- κ B p65 to the promoter-specific regions of *IL6* and *IL8* in mutant iPS-MPs (Fig 4, D and E). We referenced NF- κ B activation, proinflammatory cytokine production, and promoter binding using another isogenic pair of iPS-MPs to evaluate the consistency (Blau1-corrected and Blau1-R334W; see Fig E5 in this article's Online Repository at www.jacionline.org) and confirmed that all isogenic pairs, including Blau1, showed consistent results regarding the response to IFN- γ . Overall, our data indicate that production of proinflammatory cytokines by IFN- γ -primed iPS-MPs with mutant *NOD2* is at least partially induced through ligand-independent activation of the NF- κ B pathway.

RNA sequencing analysis reveals the involvement of inflammatory response in IFN- γ -primed iPS-MPs with a Blau syndrome-associated *NOD2* mutation

The above data revealed the relevance of the IFN- γ -induced NF- κ B activation and proinflammatory cytokine production, indicating a difference in biological status between mutant and wild-type untreated iPS-MPs in evoking an inflammatory

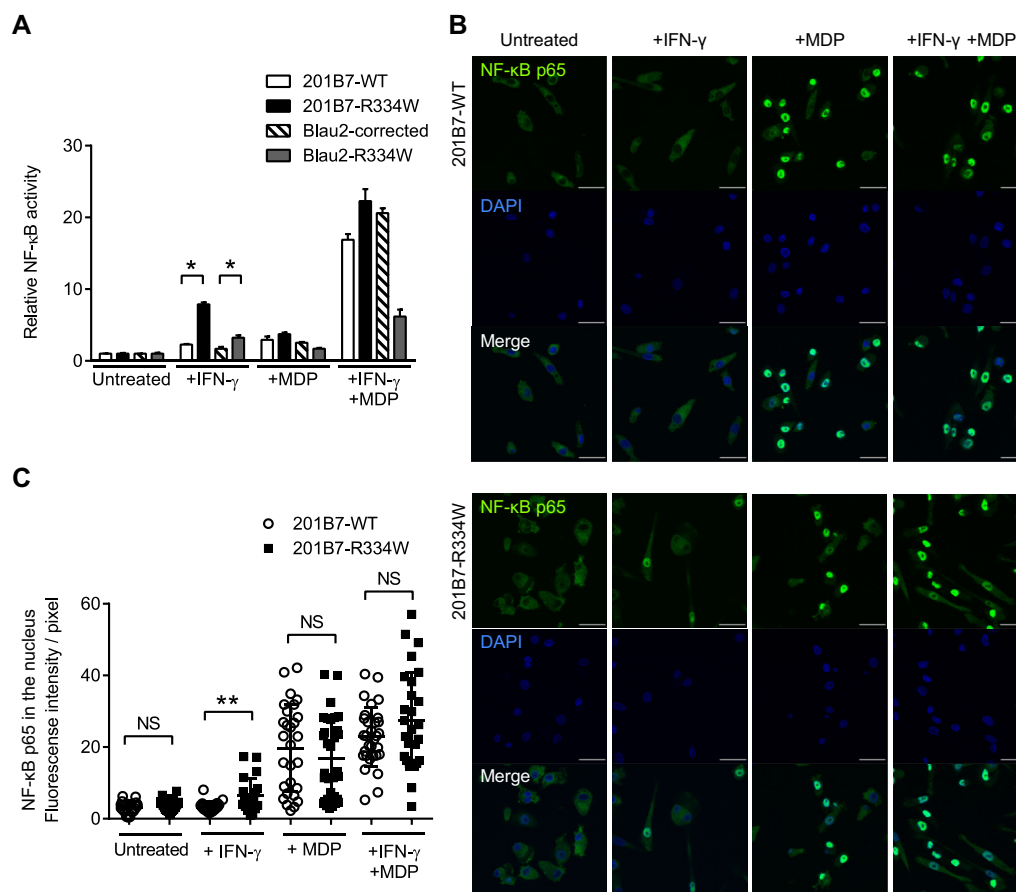


FIG 3. IFN- γ -dependent NF- κ B activation on iPS-MPs with Blau syndrome-associated mutant *NOD2*. **A**, NF- κ B activation, as evaluated by using an NF- κ B luciferase reporter system. Cells were treated with IFN- γ (50 ng/mL) for 24 hours, with the addition of MDP (10 μ g/mL) for the last 6 hours. The relative fold change from the untreated condition (set as 1) is shown. Data are presented as means $\pm$ SEMs ($n = 3$). * $P < .05$. An unpaired t test was used for statistical analysis. **B** and **C**, Evaluation of nuclear translocation of NF- κ B p65. **B**, Representative confocal images of immunofluorescence staining of NF- κ B p65 (green). Cells were treated with IFN- γ (50 ng/mL) for 24 hours, with addition of MDP (10 μ g/mL) for the last 60 minutes. Cells were stained with DAPI for visualization of the nucleus (blue). Scale bars = 40 μ m. **C**, Quantitation of the nuclear translocation of NF- κ B p65 at the single-cell level. Mean fluorescence intensity of NF- κ B p65 (green) merged with the nucleus (blue) in each cell was plotted. Bars indicate means $\pm$ SDs ($n = 20$ –35). NS, Not significant. ** $P < .01$. The Wilcoxon rank sum test was used for statistical analysis. Data are from at least 3 independent experiments.

response. To clarify the involvement of this response, we performed an RNA sequencing (RNA-seq) analysis to evaluate changes in the global transcriptional profiles of iPS-MPs. To assess the global effects of genotype and treatment on the transcriptome of iPS-MPs, we first performed a principal component analysis plot from the normalized gene counts of samples with each treatment. The transcriptome clustered predominantly by treatment, whereas the interclonal difference was minimal (Fig 5, A; for the process of normalization, see Fig E6, A–C, in this article's Online Repository at www.jacionline.org). A hierarchical clustering analysis also demonstrated that untreated and IFN- γ -treated samples grouped into distinct clusters (Fig 5, B). To extract the differentially regulated pathways between mutant and wild-type iPS-MPs, we next performed a gene set enrichment analysis (GSEA).³⁴ We confirmed that IFN- γ treatment significantly enriched the IFN- γ response pathway in all clones compared with the untreated state (see Fig E6, D and E). In mutant iPS-MPs on IFN- γ treatment, several inflammatory pathways, such as “TNFA_SIGNALING_VIA_NFKB” and

“INFLAMMATORY_RESPONSE” (false discovery rate < 0.25), were significantly enriched when compared with those in wild-type iPS-MPs (Fig 5, C). Interestingly, we noticed that similar inflammatory pathways were already upregulated in mutant iPS-MPs, even in the untreated state (Fig 5, D). These data support the idea that untreated mutant iPS-MPs are already in a distinct biological state for evoking an inflammatory response, which is maintained even after IFN- γ priming, leading to an enhanced inflammatory response, such as through proinflammatory cytokine production.

Primary macrophages primed by IFN- γ from a patient with Blau syndrome caused ligand-independent NF- κ B activation and proinflammatory cytokine production

Although the iPSC-derived isogenic monocytic cell line pairs enabled us to precisely evaluate the *in vitro* phenotype, there remains a possibility that the long-term *in vitro* culture and

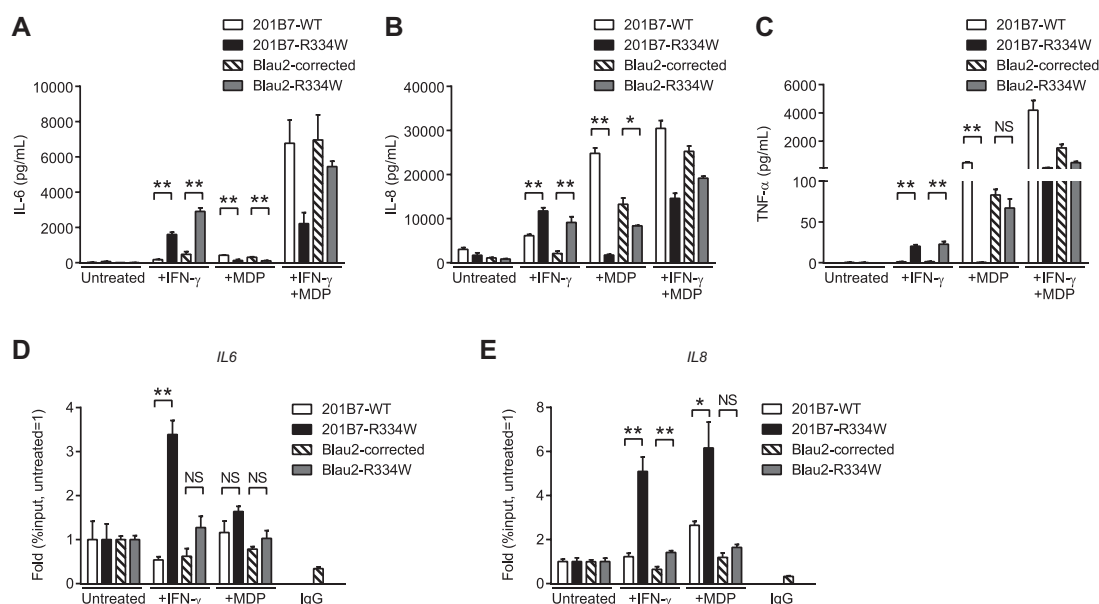


FIG 4. IFN- γ induces proinflammatory cytokine production on *NOD2*-mutated iPS-MPs by increasing binding of NF- κ B p65 to their promoter regions. **A–C**, Secretion of proinflammatory cytokines IL-6 (Fig 4, A), IL-8 (Fig 4, B), and TNF- α (Fig 4, C). Cells were treated with IFN- γ (50 ng/mL) for 24 hours, with addition of MDP (10 μ g/mL) for the last 6 hours. Data are presented as means $\pm$ SEMs ($n = 3$). NS, Not significant. * $P < .05$ and ** $P < .01$. An unpaired t test was used for the statistical analysis. **D** and **E**, Quantitative PCR analysis of ChIP samples (ChIP-qPCR) using anti-NF- κ B p65 antibody. Binding to the promoter region of *IL6* (Fig 4, D) and *IL8* (Fig 4, E) was evaluated. Cells were treated with IFN- γ (50 ng/mL) for 24 hours, with addition of MDP (10 μ g/mL) for the last 60 minutes. Data are presented as means $\pm$ SDs ($n = 3$). NS, Not significant. * $P < .05$ and ** $P < .01$. An unpaired t test was used for statistical analysis. Data are from at least 3 independent experiments.

introduction of transgenes might alter the cellular phenotype. To exclude this possibility, we investigated whether the *in vitro* phenotypes could be recapitulated with primary Mo-MPs. For this, Mo-MPs were obtained from the PBMCs of a patient with Blau syndrome with a *NOD2* R334W mutation. IFN- γ treatment upregulated the endogenous expression of *NOD2* in Mo-MPs from the control subject (Fig 6, A). Both the control subject- and patient-derived Mo-MPs demonstrated no obvious spontaneous nuclear translocation of NF- κ B when untreated (Fig 6, B and C). Similar to the observation with iPS-MPs, some of the IFN- γ -primed Mo-MPs from the patient showed NF- κ B p65 nuclear translocation without MDP, and the degree of NF- κ B p65 nuclear translocation was significantly greater in the patient-derived Mo-MPs than in those of the control subject (Fig 6, B and C). MDP stimulation promoted nuclear translocation of NF- κ B p65 in both the control subject and patient, but the degree was smaller in the patient's Mo-MPs than in those of the control subject.

We then evaluated production of proinflammatory cytokines from Mo-MPs. IFN- γ priming significantly increased IL-6, IL-8, and TNF- α production from the patient-derived Mo-MPs in a ligand-independent manner (Fig 6, D–F). Collectively, IFN- γ treatment activated the NF- κ B pathway and induced production of proinflammatory cytokines from patient-derived macrophages. These data were compatible with those from iPS-MPs, confirming the validity and usefulness of our iPSC-based modeling.

DISCUSSION

In this study we demonstrated that IFN- γ -primed human macrophages with Blau syndrome-associated mutant *NOD2*

cause ligand-independent activation of NF- κ B transcription and production of proinflammatory cytokines, such as IL-6 and IL-8. One of the main roadblocks to studying Blau syndrome-associated *NOD2* mutants has been the lack of a proinflammatory response in mutant cells *in vitro* despite autoinflammatory symptoms *in vivo*. Because both patient-derived cells and a knock-in mouse model also showed reduced proinflammatory responses, the argument remains as to whether the Blau syndrome-associated *NOD2* mutations are gain of function or loss of function. In the present study we identified IFN- γ as a specific stimulant inducing a proinflammatory response in both iPSC- and patient-derived primary macrophages. Of note, human subjects with complete IFN- γ receptor deficiency, whether IFN- γ R1 or IFN- γ R2, do not display any mature granuloma,³⁵ indicating the essential role of IFN- γ in the formation of granulomas. Our data provide important insights into the role of *NOD2* in IFN- γ -dependent granuloma formation. Although the involvement of IFN- γ signaling has been implicated in the pathology of Blau syndrome, there have been no reports proving the direct effect of IFN- γ on the function of mutant *NOD2*. Our findings revealed a previously undefined pathway for activating *NOD2*-mutated macrophages.

RNA-seq data indicated that untreated macrophages with mutant *NOD2* are already in a primed biological state to evoke an inflammatory response. Although untreated macrophages did not activate the NF- κ B pathway because of a shortage of autoactivated mutant *NOD2* protein, the upregulation of mutant *NOD2* expression by IFN- γ might cause autoactivation of *NOD2*, leading to subsequent NF- κ B activation. However, because the relationship between IFN- γ -induced proinflammatory cytokine

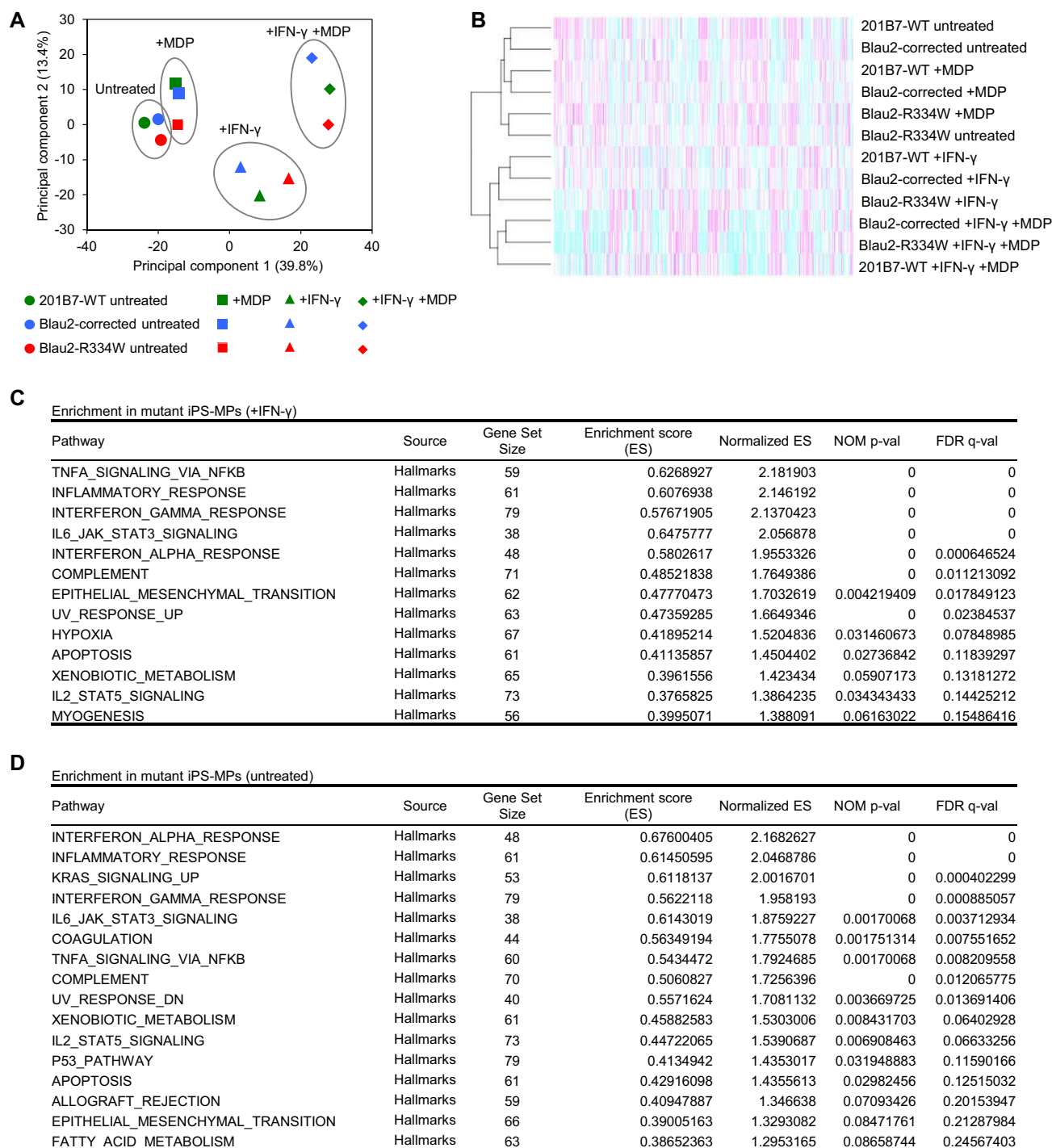


FIG 5. RNA-seq analysis of iPS-MPs. **A**, Principal component analysis based on normalized RNA-seq data. Each *symbol* indicates each clone with each treatment. **B**, Hierarchical clustering and heat map of normalized RNA-seq data. The peak transcript level is magenta, and the trough is cyan. **C** and **D**, GSEA. Gene sets of significantly (false discovery rate [FDR] < 0.25) enriched mutant iPS-MPs with each treatment (treated with IFN-γ [Fig 5, C] or untreated [Fig 5, D]) in hallmarks are shown (size filtered by 15-500).

production and granuloma formation is still largely unknown, further studies will be required to gain a comprehensive understanding of the pathophysiology of Blau syndrome.

A response to MDP subsequently reduced the level of proinflammatory cytokine production in mutant iPS-MPs, which

is consistent with the previous findings in the mouse model¹⁵ and in the patients' PBMCs.¹⁷ Although MDP treatment did not lead to reduced cytokine production in the primary Mo-MPs in our study, one of the fundamental problems in the functional analysis of human primary cells is the large degree of variance, even

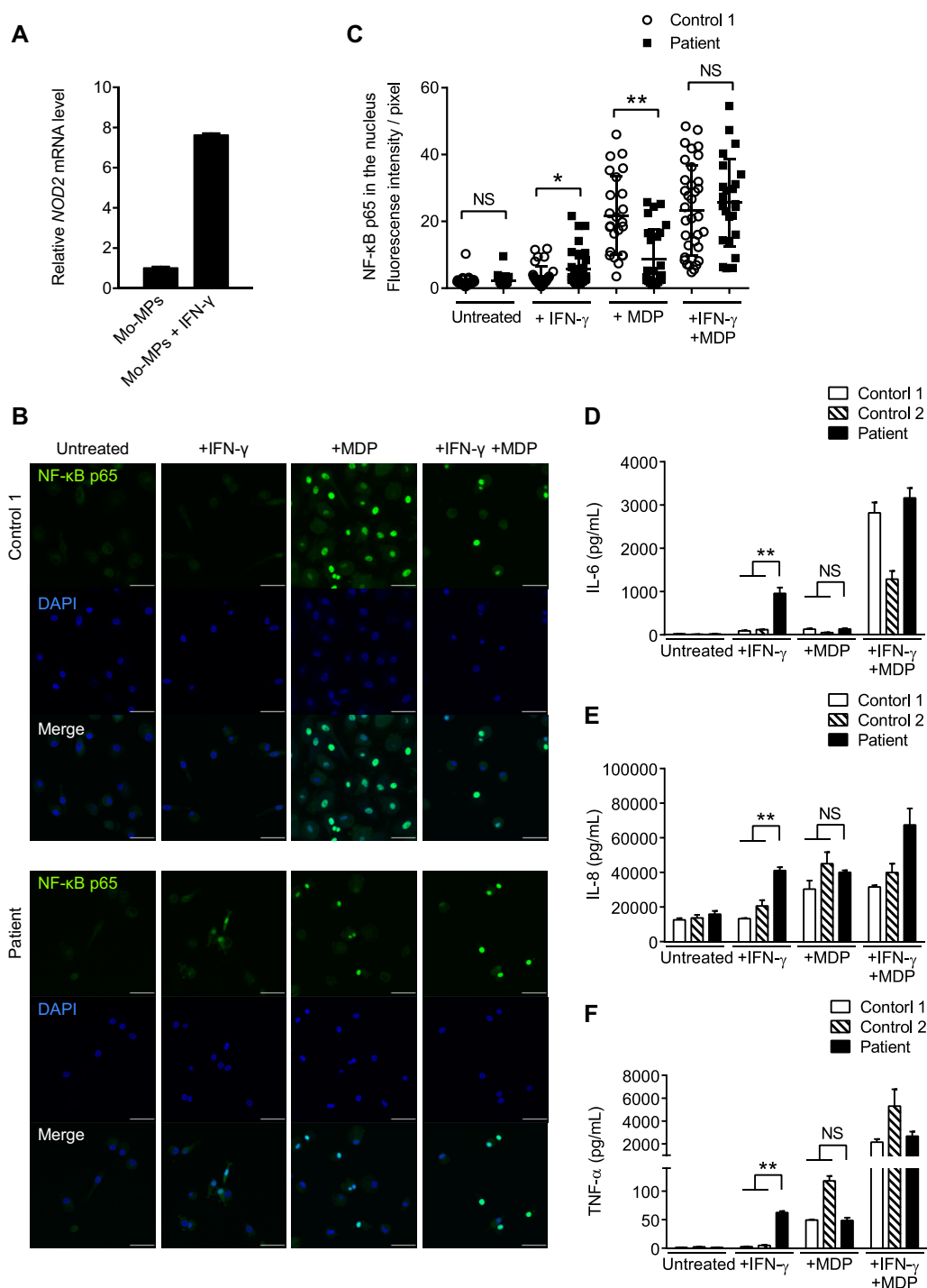


FIG 6. IFN- γ -dependent NF- κ B activation and proinflammatory cytokine production in patient-derived Mo-MPs. **A**, Quantitative RT-PCR validation of *NOD2* expression in control Mo-MPs. Cells were treated with IFN- γ (50 ng/mL) for 24 hours. Relative fold change from untreated condition (set as 1) is shown. Data are presented as means $\pm$ SDs ($n = 3$). **B** and **C**, Evaluation of nuclear translocation of NF- κ B p65. Fig 6, **B**, Representative confocal images of immunofluorescence staining of NF- κ B p65 (green). Cells were treated with IFN- γ (50 ng/mL) for 24 hours, with addition of MDP (10 μ g/mL) for the last 60 minutes. Cells were stained with DAPI for visualization of the nucleus (blue). Scale bars = 40 μ m. Fig 6, **C**, Quantitation of nuclear translocation of NF- κ B p65 at the single-cell level. Mean fluorescence intensity of NF- κ B p65 (green) merged with nucleus (blue) in each cell was plotted. Bars indicate means $\pm$ SDs ($n = 18$ -34). NS, Not significant. * $P < .05$ and ** $P < .01$. A Wilcoxon rank sum test was used for statistical analysis. **D-F**, Secretion of proinflammatory cytokines IL-6 (Fig 6, **D**), IL-8 (Fig 6, **E**), and TNF- α (Fig 6, **F**). Cells were treated with IFN- γ (50 ng/mL) for 24 hours, with addition of MDP (10 μ g/mL) for the last 6 hours. Data are presented as means $\pm$ SEMs ($n = 3$). NS, Not significant. * $P < .05$. An unpaired t test was used for the statistical analysis. Data are from 2 independent experiments.

among healthy control subjects, which is shown in Fig 6, D-F. Cell-derived iPSCs share the same genomic backgrounds as primary blood cells, but the *NOD2* genotypes differ between isogenic pairs, which enables us to exclude any interpersonal variance. The loss of response to MDP in cytokine production might be associated with the pathogenesis of Blau syndrome because granulomas are sometimes caused by the inability to eliminate foreign bodies and pathogens.³⁶ In contrast to the findings of the cytokine production assay, MDP stimulation activated NF- κ B pathway in both mutant and wild-type clones and tended to enhance recruitment of the NF- κ B p65 subunit to promoter regions of *IL6* and *IL8* (Fig 4, D and E). Although the underlying mechanism for this paradoxical response remains to be elucidated, our data indicate the possible existence of an alternative suppressive pathway activated by mutant *NOD2* or another pathway through MDP for producing proinflammatory cytokines independent of *NOD2*.

Based on our observation from iPS-MPs and patient-derived Mo-MPs, blocking IFN- γ signaling in patients with Blau syndrome can be a potential treatment for managing chronic inflammation. For example, the Janus kinase inhibitors tofacitinib, baricitinib, and ruxolitinib are already clinically available. However, before this concept can be applied in a clinical setting, a number of issues remain to be solved.

First, we do not have direct evidence to prove that the IFN- γ pathway is activated in patients with Blau syndrome. For this, further studies investigating primary samples of patients, including blood and pathologic specimens, will be required to confirm the upregulation of IFN- γ signatures.

Second, because there are other priming signals, such as TNF- α , LPS, and other Toll-like receptor ligands, known to upregulate *NOD2* expression,²²⁻²⁴ IFN- γ -dependent priming of macrophages might not be the principal condition for evoking autoinflammation. In the current study we did not evaluate the effects of these signals because of the technical difficulty of distinguishing the Toll-like receptor- or TNF receptor-dependent NF- κ B activation from the *NOD2*-dependent one. However, IFN- γ usually does not transduce signals activating the NF- κ B pathway, enabling us to investigate the role of IFN- γ as a priming signal.

Third, we must consider other effects of IFN- γ associated with a substantial immune response and other signal pathways. To avoid these issues, we must suppress the specific pathways induced by mutant *NOD2* when applying this therapy in treatments.

In conclusion, by investigating the functions of macrophages under controlled conditions with a comprehensive isogenic iPSC panel with a Blau syndrome-associated *NOD2* mutation, we were able to perform strictly controlled cellular assays and found that IFN- γ provokes a series of aberrant inflammatory responses in mutant macrophages. Because we focused mainly on *NOD2*-dependent NF- κ B pathway activation using macrophages in this study, further studies exploring alternative *NOD2*-dependent pathways or with other cell types are required to clarify the comprehensive pathophysiology of Blau syndrome. Phenotypes related to genotypes other than R334W should be evaluated as well. Through the Blau syndrome study, we expect that our iPSC model will also provide an opportunity for elucidating the previously unknown mechanisms of granuloma formation in patients with other diseases.

We thank the patients and family for their cooperation. We also thank Drs Satoru Senju (Kumamoto University, Kumamoto, Japan) and Hiroyuki Miyoshi (RIKEN Bio Resource Center, Tsukuba, Japan) for providing lentiviral vectors. We are grateful to Ryunosuke Ikeda and Tomoyo Ukai for their technical assistance. We also thank Yoshinori Hasegawa and Osamu Ohara for supporting RNA-seq analysis. We also thank Harumi Watanabe for administrative assistance.

Clinical implications: Our observation of an aberrant inflammatory response in *NOD2* mutant macrophages highlights the significance of IFN- γ -dependent autoinflammation in the pathophysiology of Blau syndrome as a potential therapeutic target.

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METHODS

Control iPSC lines

The human iPSC line 201B7^{E1} was kindly provided by Dr Shinya Yamanaka (Kyoto University, Kyoto, Japan).

Establishment and maintenance of iPSCs

iPSCs were generated from blood cells, as described previously.^{E2,E3} Briefly, blood cells were isolated from peripheral blood by using a BD Vacutainer (BD Biosciences, San Jose, Calif) in accordance with the manufacturer's instructions. The PBMCs were cultured in StemSpan-ACF medium (STEMCELL Technologies, Vancouver, British Columbia, Canada) containing stem cell factor (SCF; 100 ng/mL), thyroperoxidase (TPO; 100 ng/mL), Flt ligand (100 ng/mL), IL-6 (50 ng/mL), and IL-3 (10 ng/mL) for 5 days before transfection. SCF, TPO, Flt ligand, IL-6, and IL-3 were purchased from R&D Systems (Minneapolis, Minn). Episomal plasmids (3 µg) mixed with pCE-hOCT3/4, pCE-hSK, pCE-hUL, pCE-mp53DD, and pCXB-EBNA1 were transfected into 1×10^6 cultured PBMCs by using a Nucleofector 2b device with an Amara Human CD34 cell Nucleofector kit. The electroporated cells were plated onto tissue-culture plates coated with the Laminin511-E8 fragment iMatrix-511 (Nippi, Tokyo, Japan) containing StemSpan-ACF medium with the same cytokines as described above. The culture medium was gradually changed to StemFit AK03 medium (Ajinomoto, Tokyo, Japan). Two to 3 weeks after transduction, individual colonies were isolated and expanded. The iPSCs were cultured on LN511E8-coated tissue-culture plates with StemFit AK03 medium at 37°C with 5% CO₂ and 21% O₂ and passaged through dissociation into single cells by using TrypLE Select (Life Technologies, Gaithersburg, Md), as described previously.^{E2}

Characterization of established iPSCs

Chromosomal G-banding analyses were performed at the Nihon Gene Research Laboratory (Miyagi, Japan). For the teratoma formation assay, approximately 2×10^6 cells were injected subcutaneously into the dorsal flank of immunocompromised NOD/scid/cc^{null} mice (Central Institute for Experimental Animals, Kawasaki, Japan). Masses were excised 9 to 14 weeks after injection and fixed with 4% paraformaldehyde in PBS(−). Paraffin-embedded tissues were sliced and stained with hematoxylin and eosin. Slides were examined with the BIOREVO BZ-9000 microscope (Keyence, Osaka, Japan). A PlanApo 10×/0.45 objective (Nikon, Tokyo, Japan) and the BZ-II Viewer software program (Keyence) were used for image acquisition.

Flow cytometry

Surface markers on hematopoietic cells were evaluated with a MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany). Primary antibodies for the analysis were as follows: CD11b–fluorescein isothiocyanate (Beckman Coulter, Brea, Calif), CD45–fluorescein isothiocyanate (BD Pharmingen, San Diego, Calif), CD34–phycoerythrin (Beckman Coulter), CD86–phycoerythrin (BD Pharmingen), CD11c–allophycocyanin (BD Pharmingen), and CD14–allophycocyanin (Beckman Coulter). Dead cells were excluded by using 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich, St Louis, Mo) staining. Data analyses were performed with FlowJo software (TreeStar, Ashland, Ore).

May-Giemsa staining

Cells were seeded onto glass slides by using PLATINUM PRO (Matsunami, Osaka, Japan) and stained with May-Grunwald and Giemsa staining solution (Merck Millipore, Darmstadt, Germany) in accordance with the manufacturer's instructions. The slides were examined with a BIOREVO BZ-9000 (Keyence). A PlanApo 40×/0.95 objective (Nikon) and the BZ-II Viewer software program (Keyence) were used for image acquisition.

Hematopoietic cell differentiation

Hematopoietic cell differentiation was performed in accordance with previously described protocols, with some modifications.^{E4,E5} Briefly, 4×10^2 to 1.6×10^3 undifferentiated iPSCs were first disseminated onto a 6-well

tissue culture dish coated with growth factor–reduced Matrigel in mTeSR1 medium. When individual colonies grew to approximately 500 µm in diameter, differentiation was started by adding BMP4 (80 ng/mL, R&D systems). Cells were cultured at 37°C with 5% CO₂ and 5% O₂ during differentiation. On day 2, the medium was replaced with Essential 6 (Life Technology) containing vascular endothelial growth factor (80 ng/mL), basic fibroblast growth factor (50 ng/mL), SCF (50 ng/mL), and SB431542 (2 µmol/L). On day 4, the medium was replaced with Stemline 2 (Sigma-Aldrich) containing SCF (50 ng/mL), TPO (5 ng/mL), IL-3 (50 ng/mL), macrophage colony-stimulating factor (M-CSF; 50 ng/mL), and Flt-3 ligand (50 ng/mL) for monocytic cell differentiation. On day 12, the cytokines were switched to the combination of M-CSF (50 ng/mL), Flt-3 ligand (50 ng/mL), and GM-CSF (25 ng/mL). BMP4, vascular endothelial growth factor, SCF, TPO, IL-3, M-CSF, GM-CSF, and Flt ligand were purchased from R&D Systems. Basic fibroblast growth factor, CHIR99021, was purchased from Wako Pure Chemical Industries (Osaka, Japan). SB431542 was purchased from Merck Millipore.

Sanger sequencing

Genomic DNA was extracted from the cells by using the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) or PureLink Genomic DNA Kits (Invitrogen, Carlsbad, Calif) and subjected to PCR amplification with primers for *NOD2* genotyping (Table E1). After treatment with USB ExoSAP-IT (Affymetrix, Santa Clara, Calif), the PCR products were used for Sanger sequencing. Sequencing was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, Calif), and the subsequent products were purified with the BigDye XTerminator Purification Kit (Applied Biosystems). The 3500xL sequencer (Applied Biosystems) was used for analyses. Data were analyzed with the CodonCode Aligner software program (CodonCode, Centerville, Mass).

Generation of iPS-MLs

iPS-MLs were established, as previously described.^{E6} In brief, lentiviral constructs encoding *BMI1*, *cMYC*, and *MDM2* in the CSII-EF-RfA vector were kindly provided by Drs Satoru Senju (Kumamoto University, Kumamoto, Japan) and Hiroyuki Miyoshi (RIKEN Bio Resource Center, Tsukuba, Japan). Floating hematopoietic cells differentiated into the monocytic lineage from iPSCs were collected on days 15 to 18. Cells were transfected with the lentiviral vectors and subsequently cultured in StemPro-34 serum-free medium containing 2 mmol/L L-glutamine in the presence of M-CSF (50 ng/mL). For macrophage differentiation, culture conditions were changed to RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS and M-CSF (100 ng/mL) for 7 to 9 days. For polarization of macrophages, IL-4 (20 ng/mL) or IFN-γ (20 ng/mL) was added for the last 24 hours. We then collected adherent macrophages by treating them with Accumax (Innovative Cell Technologies, San Diego, Calif) and used the collected cells for subsequent experiments.

Generation of corrected or knocked in iPSC clones

We corrected the mutant *NOD2* gene by using the CRISPR-Cas9 system. The guide RNA (gRNA) sequence was designed on the coding sequence of *NOD2* in intron 3 (CCTTCAGTTATGTCAGCGTCCCC). gRNA and Cas9 vectors were purchased from Takara Bio (Shiga, Japan). Clones maintained under feeder-free conditions were dissociated into a single cell. Vectors expressing gRNA and Cas9 were then introduced with a NEPA21 electroporator (Nepagene, Chiba, Japan). Two days after transfection, cells were incubated with puromycin (0.5–1 µg/mL) for 24 to 48 hours or neomycin (50 µg/mL) for 1 week. Surviving colonies were picked up 1 week after the start of selection, passaged and expanded several times, and frozen. DNA was extracted from the cells and genotyped by using Sanger sequencing, as described above. Vectors expressing Cre recombinase were then introduced by HuGENE HD Transfection Reagent (Promega, Madison, Wis) to excise the loxP-flanked drug selection cassette. For construction of the C-terminal 3×FLAG sequence knocking-in allele, the gRNA sequence was designed on the coding sequence of *NOD2* in exon 12 (CTTGCTTTGAAGTCTCCGGGAGG). Vectors were transfected, and cells were cultured, as described above.

Quantitative PCR

RNA samples were prepared with the AllPrep DNA/RNA Mini Kit (Qiagen) or RNeasy Mini Kit (Qiagen) and subjected to reverse transcription with a PrimeScript RT Master Mix (Takara Bio). All procedures were performed in accordance with the manufacturer's instructions. Quantitative PCR (qPCR) was performed on the StepOnePlus Real-Time PCR System (Applied Biosystems). For detection of transgenes, DNA was subjected to qPCR, and SYBR Premix ExTaqII (Takara Bio) was used for detection. Relative quantification values are shown. For quantification of the expression of pluripotent stem cell-associated genes (Fig E1, C), qPCR was performed with cDNA and the Fast Advanced Master Mix (Applied Biosystems). Data were processed by using the $\Delta\Delta$ cycle threshold method, and the relative quantities to the control 201B7 line are shown. The primer sequences are shown in Table E1.

Immunoblotting

Cell lysates were lysed for 30 minutes on ice in RIPA buffer (Wako Pure Chemical Industries) plus proteinase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). After being centrifuged at 15,000g for 20 minutes at 4°C, supernatants were collected, and dithiothreitol was added. The lysates were incubated at 95°C for 5 minutes. Proteins were then separated by using SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 3% nonfat milk in Tris-buffered saline plus 0.1% Tween 20. Immunoblotting was performed with FLAG M2 mouse mAb (1:1000, Sigma-Aldrich) and horseradish peroxidase-conjugated anti-mouse IgG antibody (1:3000; Santa Cruz Biotechnology, Santa Cruz, Calif) and detected with a LAS4000 (Fujifilm, Tokyo, Japan).

NF- κ B Dual Luciferase assay

iPS-MLs were transfected with pGreenFire NF- κ B transcription reporter lentivector (System Biosciences, Mountain View, Calif) inserted with the Renilla gene under the CMV promoter as an internal control. iPS-MLs were differentiated into macrophages, as described above. Cells were cultured with or without 50 ng/mL of IFN- γ (R&D Systems) for 24 hours, with addition of 10 μ g/mL MDP (Sigma-Aldrich) for the last 6 hours. The NF- κ B activity was then measured by using the Dual-Luciferase Reporter Assay System (Promega).

Immunofluorescence

Macrophages were seeded into multiwell glass-bottom dishes (Matsunami) and cultured with or without 50 ng/mL IFN- γ for 24 hours, with addition of 10 μ g/mL of MDP for the last 60 minutes. Cells were then fixed with 4% paraformaldehyde in PBS(−) for 30 minutes. Permeabilization and blocking were performed with blocking buffer (Blocking One [Nacalai Tesque] with 0.1% Tween20 [Santa Cruz Biotechnology]) for 30 minutes. Samples were incubated with anti-NF- κ B p65 rabbit mAb (1:400; Cell Signaling Technologies, Danvers, Mass) in blocking buffer overnight at 4°C and subsequently incubated with Alexa Fluor 488 goat anti-mouse IgG (1:1000; Cell Signaling Technologies) for 30 minutes. The nuclei were stained with DAPI (1:100) in PBS(−). The cells were visualized with a FluoVIEW10i confocal microscope (Olympus, Tokyo, Japan) and analyzed with ImageJ software (National Institutes of Health, Bethesda, Md).

Cytokine assay

Concentrations of cytokines in the culture supernatants were measured by using LEGENDplex Multi-Analyte Flow Assay Kits (BioLegend, San Diego, Calif) in accordance with the manufacturer's instructions. Quantification was done with a MACSQuant Analyzer (Miltenyi Biotec). iPS-MLs were differentiated into macrophages, as described above. For stimulation, cells were treated with IFN- γ (50 ng/mL) for 24 hours, and MDP (10 μ g/mL) was added to the medium for the last 6 hours when indicated.

Isolation of PBMCs and macrophage differentiation

After receiving informed consent, peripheral blood was drawn from a patient and 2 healthy volunteers. PBMCs were isolated by using Lymphoprep

(STEMCELL Technologies). CD14⁺ monocytic cells were sorted with the EasySep Human CD14 Positive Selection Kit (STEMCELL Technologies). Cells were differentiated into macrophages, as described above, and the differentiated macrophages were then subjected to an analysis.

ChIP-qPCR

After stimulation, cells were cross-linked in 1% formaldehyde for 10 minutes at room temperature. The nuclear lysates were sonicated with a sonicator (Misonix, Farmingdale, NY). After being centrifuged at 15,000g for 30 minutes at 4°C, the supernatants were collected. The clarified lysates were divided in half and treated overnight at 4°C with 10 μ g of anti-NF- κ B p65 (C-20) rabbit polyclonal antibody (Santa Cruz Biotechnology) or rabbit IgG polyclonal-isotype control (Abcam, Cambridge, United Kingdom) preincubated with Dynabeads M-280 Sheep anti-Rabbit IgG (Thermo Fisher Scientific, Waltham, Mass). The protein-DNA complexes were captured on a magnet and eluted with TE buffer containing 1% SDS at 65°C. After cross-link reversal and purification with MinElute PCR Purification Kit (Qiagen), quantitative PCR was performed, as described above. The primer sequences are described in Table E1.

Cell viability assay

Cell viability was measured with a Countess Automated Cell Counter (Invitrogen). For stimulation, cells were treated with IFN- γ (50 ng/mL) for 24 hours, and MDP (10 μ g/mL) was added to the medium for the last 6 hours when indicated.

RNA-seq and data normalization

RNA-seq analysis was performed at the Kazusa DNA Institute, as previously described.^{E7} RNA-seq libraries were prepared by using a SureSelect Strand Specific RNA Library Preparation kit (Agilent Technologies, Santa Clara, Calif). Sequencing was performed on an Illumina HiSeq1500 with a HiSeq Rapid SBS kit v2 (Illumina, San Diego, Calif) in a 50-base single-end mode. The mRNA profiles were compiled with the Cufflinks (ver. 2.2.1) software program and expressed as fragments per kilobase of exon model per million mapped fragments. For the analysis, the data sets filtered by the criterion requiring fragments per kilobase of exon model per million mapped fragment values of greater than 0 in at least 1 sample were first subjected to variance-stabilizing transformation and interquartile range filtering. Then, upper-quartile normalization followed by remove unwanted variation (RUV) normalization regarding each 3 lines of treated and untreated samples as a replicated group (RUV), modeling $k = 1$ factors of unwanted variation, was performed to remove unwanted variations.^{E8,E9} Upper-quartile and RUV normalization was conducted by using the open-source R package DeSeq2 and RUVSeq, the source code of which is freely available through the Bioconductor Project.

GSEA

GSEA (<http://software.broadinstitute.org/gsea/index.jsp>) was used to estimate the biological signatures. Normalized data sets were applied for each analysis by using the Molecular Signatures Database (version 5.1; Hallmark gene sets, size filters set at 15-500).

GSE accession numbers

RNA-seq data sets were deposited in the GEO database and can be accessed with GEO accession number GSE98454.

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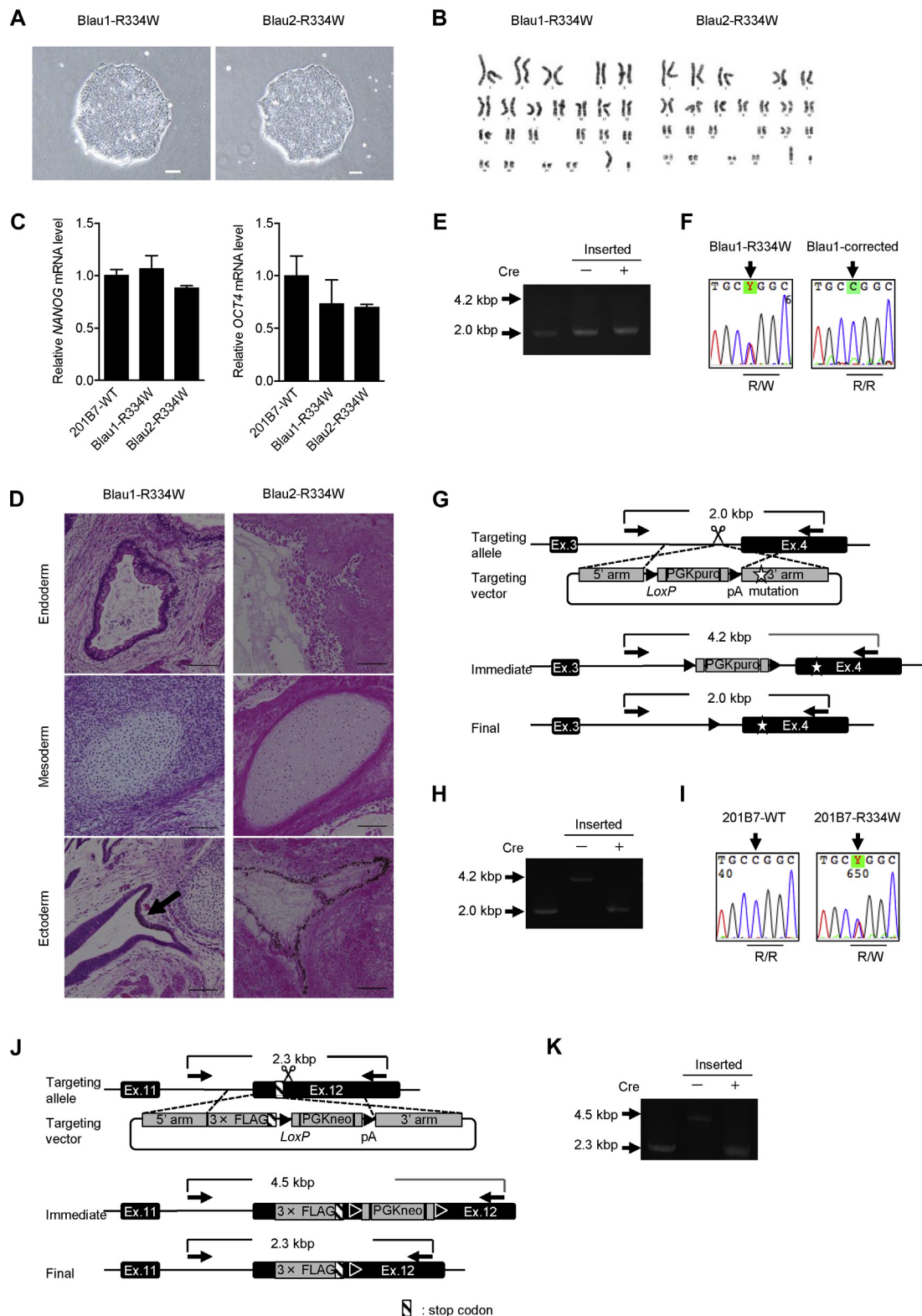


FIG E1. Characterization of iPSCs. **A**, Morphology of iPSCs. Scale bars = 100 μ m. **B**, Karyotype analysis of representative clones. **C**, Quantitative RT-PCR validation of expression of the pluripotent stem cell-associated genes *NANOG* and *OCT4*. **D**, Representative images of the teratoma formation assay. Arrow indicates melanin. Scale bars = 100 μ m. **E**, Validation of the corrected allele by means of genomic PCR in Blau1-R334W iPSCs. **F**, Representative chromatogram of Sanger sequencing of *NOD2*. **G**, Schematic illustration of the stepwise gene knock-in of *NOD2* R334W mutation into control 201B7 iPSC clones using the CRISPR-Cas9 system. Arrows indicate the PCR primers used in Fig E1. **H**, Validation of the knocked-in allele by using genomic PCR. **I**, Representative chromatogram of Sanger sequencing of *NOD2*. **J**, Scheme for introducing the 3 $\times$ FLAG tag into the C-terminus of *NOD2* by using the CRISPR-Cas9 system. Arrows indicate PCR primers used in Fig E1. **K**, Validation of the knocked-in allele by using genomic PCR.

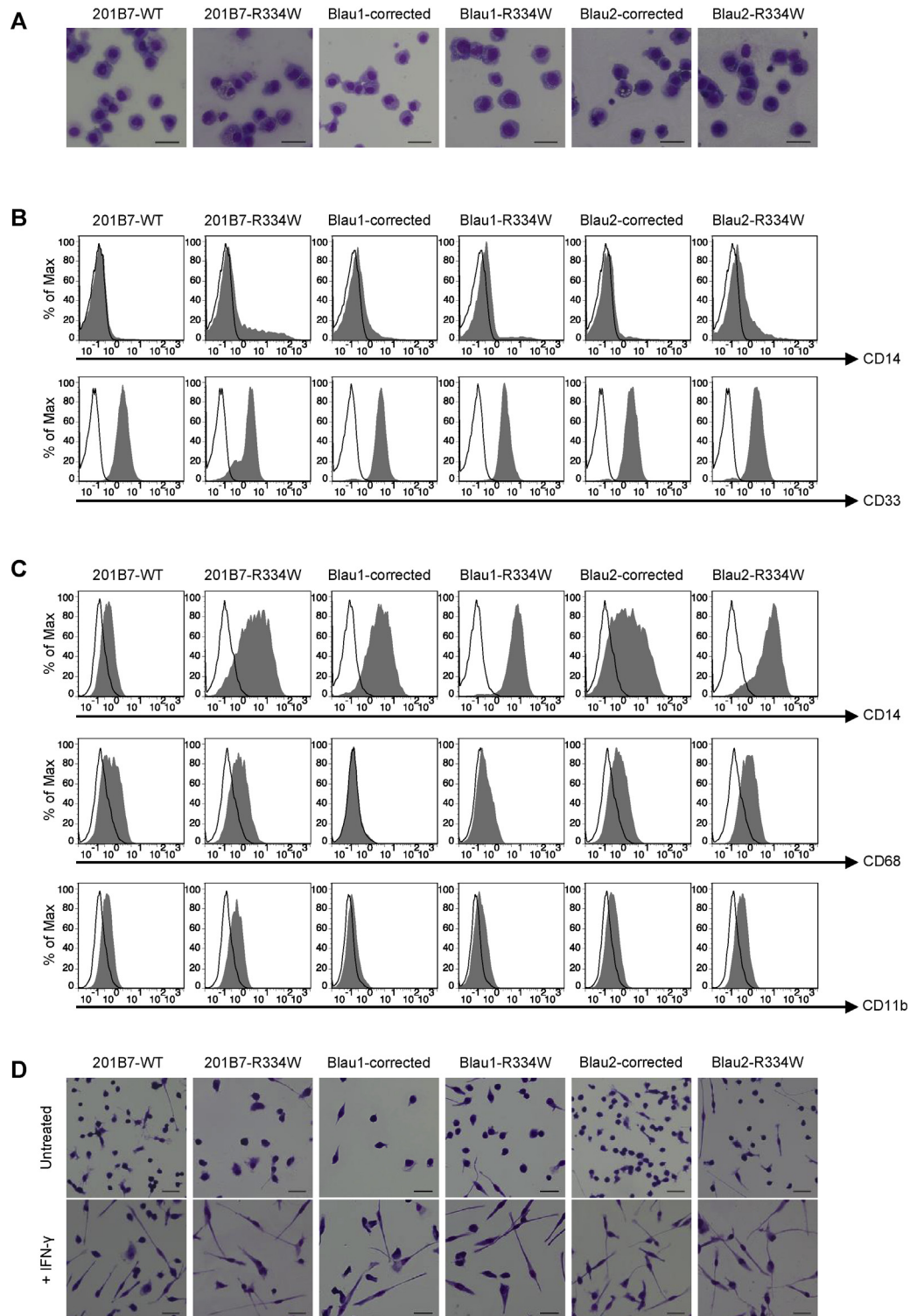


FIG E2. Characterization of iPS-MLs and iPS-MPs. **A**, Representative May-Giemsa images of iPS-MLs. Scale bars = 40 μ m. **B**, Flow cytometric analysis of iPS-MLs. The white area shows the isotype control. **C**, Flow cytometric analysis of untreated iPS-MPs. The white area shows the isotype control. **D**, Representative May-Giemsa images of iPS-MPs. Scale bars = 40 μ m. Cells were treated with IFN- γ (50 ng/mL) for 24 hours.

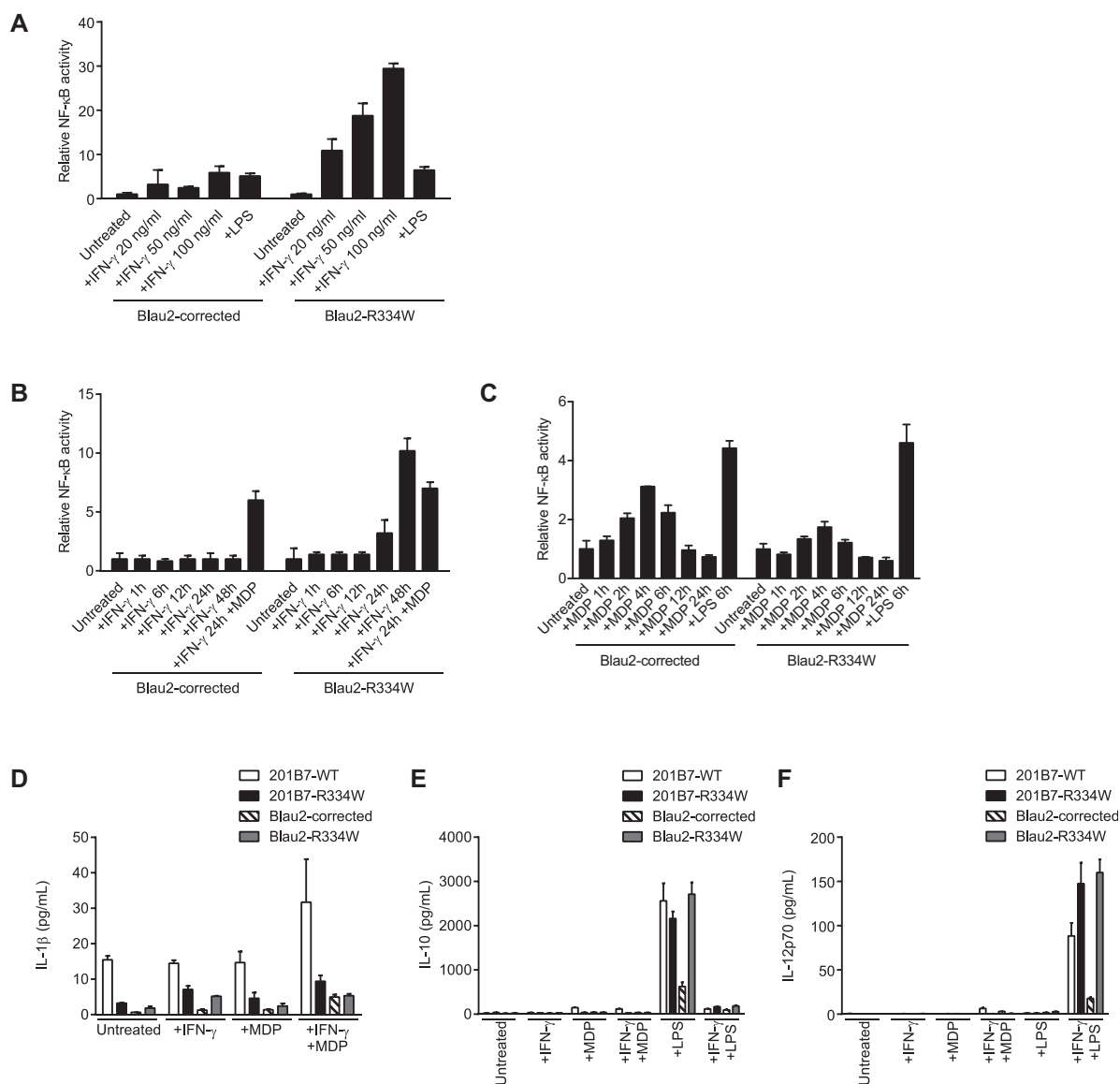


FIG E3. Response of iPS-MPs to IFN- γ . **A** and **B**, Dose and time dependence of NF- κ B activation by IFN- γ evaluated by using an NF- κ B luciferase reporter system. Fig E3, **A**, iPS-MPs were treated with the indicated dose of IFN- γ for 24 hours or LPS (100 ng/mL) for 6 hours ($n = 3$). Fig E3, **B**, iPS-MPs were treated with IFN- γ (50 ng/mL) for the indicated time periods. MDP (10 μ g/mL) was added for the last 6 hours, when indicated ($n = 3$). The relative fold change from the untreated condition (set as 1) is shown. **C**, Time course of NF- κ B activation by MDP evaluated with an NF- κ B luciferase reporter system. iPS-MPs were treated with MDP (10 μ g/mL) for the indicated time periods or LPS (100 ng/mL) for 6 hours ($n = 3$). Relative fold change from the untreated condition (set as 1) is shown. **D-F**, Secretion of cytokines IL-1 β (Fig E3, **D**), IL-10 (Fig E3, **E**), and IL-12p70 (Fig E3, **F**). Cells were treated with IFN- γ (50 ng/mL) for 24 hours, with addition of MDP (10 μ g/mL) or LPS (100 ng/mL) for the last 6 hours ($n = 3$). Fig E3, **A-F**, Data are presented as means $\pm$ SEMs. Data are from at least 3 independent experiments.

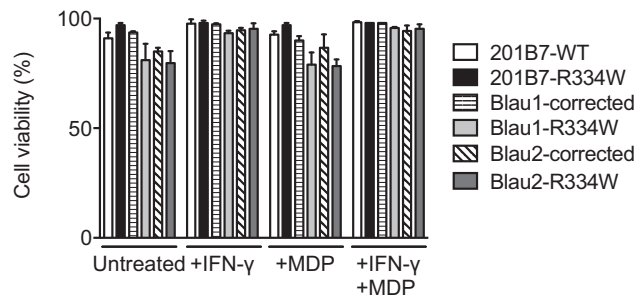


FIG E4. Cell viability of iPS-MPs. Cells were treated with IFN- γ (50 ng/mL) for 24 hours, with addition of MDP (10 μ g/mL) for the last 6 hours ($n = 3$). Data are presented as means $\pm$ SDs. Data are from at least 3 independent experiments.

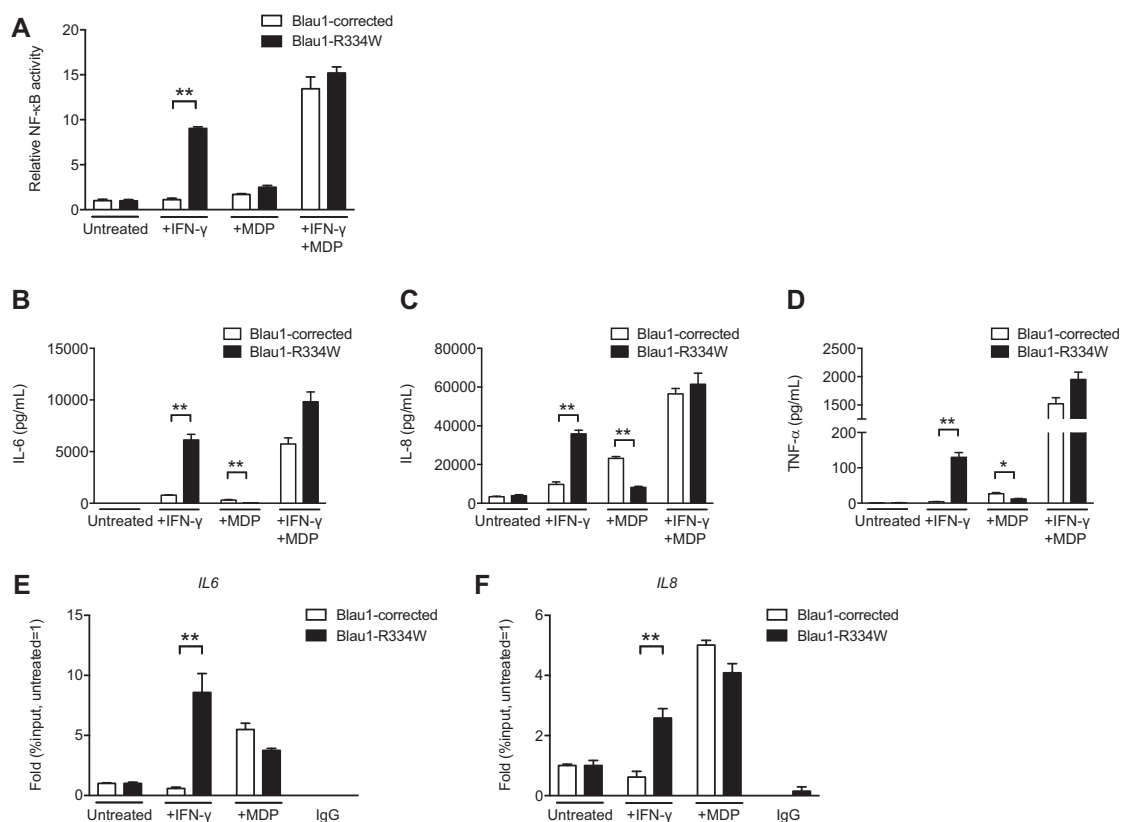


FIG E5. Ligand-independent NF-κB activation and proinflammatory cytokine production on iPS-MPs with Blau syndrome-associated mutant *NOD2*. **A**, NF-κB activation as evaluated by using an NF-κB luciferase reporter system. Cells were treated with IFN-γ (50 ng/mL) for 24 hours, with addition of MDP (10 μg/mL) for the last 6 hours. The relative fold change from the untreated condition (set as 1) is shown. Data are presented as means ± SEMs (n = 3). **B-D**, Secretion of proinflammatory cytokines IL-6 (Fig E5, **B**), IL-8 (Fig E5, **C**), and TNF-α (Fig E5, **D**). Cells were treated with IFN-γ (50 ng/mL) for 24 hours, with addition of MDP (10 μg/mL) for the last 6 hours. Data are presented as means ± SEMs (n = 3). **E** and **F**, qPCR analysis of ChIP-qPCR using anti-NF-κB p65 antibody. Binding to the promoter region of *IL6* (Fig E5, **E**) and *IL8* (Fig E5, **F**) was evaluated. Cells were treated with IFN-γ (50 ng/mL) for 24 hours, with addition of MDP (10 μg/mL) for the last 60 minutes. Data are presented as means ± SDs (n = 3). Fig E5, **A-F**, **P* < .05 and ***P* < .01. An unpaired *t* test was used for statistical analysis. Data are from at least 3 independent experiments.

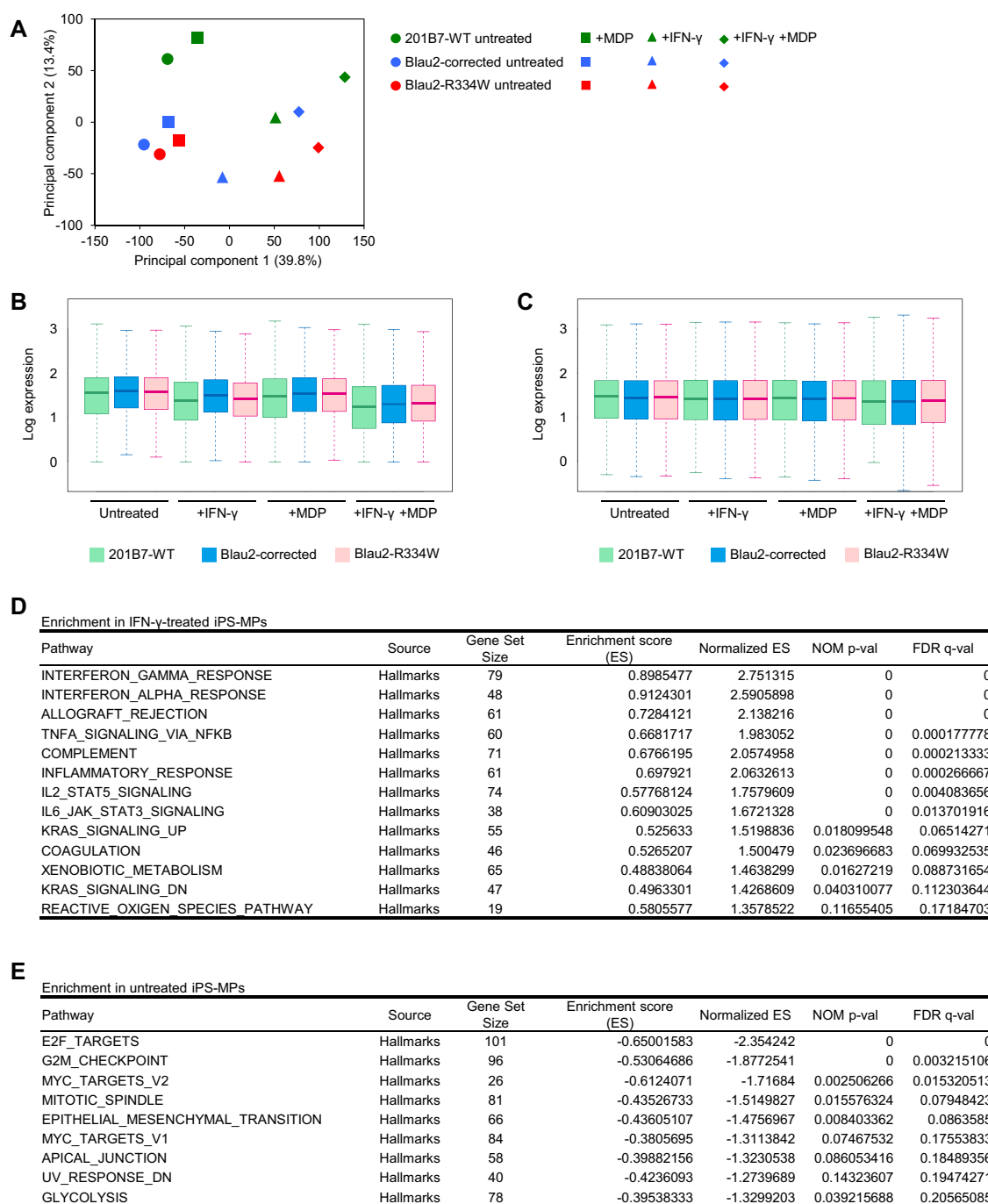


FIG E6. RNA-seq analysis of iPS-MPs. **A**, Principal component analysis based on RNA-seq data before normalization. Each *symbol* indicates each clone with each treatment. **B** and **C**, Box plots of relative log expression for unnormalized (Fig E6, *B*) and normalized (Fig E6, *C*) counts. The bottom and top of the box indicate the first and third quartiles, respectively; *inside line* indicates the median. **D** and **E**, GSEA. Gene sets of significantly (false discovery rate [*FDR*] < 0.25) enriched in IFN-γ-treated iPS-MPs from all clones (Fig E6, *D*) and untreated (Fig E6, *E*) are shown (size filtered by 15-500).

TABLE E1. List of primers

<i>NOD2</i> genotyping	Sequence 1	Sequence 2
<i>NOD2</i> PCR	AACTAAACTCTGACACAGGAAATGGTTT	AGCAGGTACATATCTGTAGTGGTCTTT
<i>NOD2</i> sequencing	AGAGAAGCCCTTGAGGTTGAACT	
qPCR for <i>NOD2</i> expression	Sequence 1	Sequence 2
<i>NOD2</i>	CTCCATGGCTAAGCTCCTTG	CACACTGCCAATGTTGTTCC
<i>GAPDH</i>	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
qPCR for ChIP	Sequence 1	Sequence 2
<i>IL6</i>	CCCTCACCCCTCCAACAAAGATTTAT	GCCTCAGACATCTCCAGTCCTATAT
<i>IL8</i>	AGCACTCCATAAGGCACAAAC	AAATCAGGAAGGCTGCCAAG
qPCR for pluripotent stem cell-associated genes		
<i>NANOG</i>	Hs02387400_g1	NANOG FAM-MGB
<i>OCT4</i>	Hs00999634_gH	POU5F1 FAM-MGB
<i>GAPDH</i>	Hs02758991_g1	GAPDH VIC-MGB

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

TABLE E2. Characterization of the iPSCs from patients with Blau syndrome

iPSC line	Karyotype	Residual plasmid	Expression of pluripotency-associated TFs (<i>NANOG</i> , <i>OCT4</i>)	<i>NOD2</i> mutations	Reprogramming methods	Teratoma formation
Blau1						
Blau1A	Normal	Checked	Yes	Yes	Episomal	Yes
Blau1B	Normal	Checked	Yes	Yes	Episomal	No experiment
Blau1C	Normal	Checked	Yes	Yes	Episomal	No experiment
Blau1D	Normal	Checked	Yes	Yes	Episomal	No experiment
Blau1E	Normal	Checked	Yes	Yes	Episomal	Yes
Blau1F	Normal	Checked	Yes	Yes	Episomal	No experiment
Blau2						
Blau2A	Normal	Checked	Yes	Yes	Episomal	No experiment
Blau2B	Normal	Checked	Yes	Yes	Episomal	Yes
Blau2C	Normal	Checked	Yes	Yes	Episomal	Yes
Blau2D	Normal	Checked	Yes	Yes	Episomal	No experiment
Blau2E	Normal	Checked	Yes	Yes	Episomal	No experiment
Blau2F	Normal	Checked	Yes	Yes	Episomal	No experiment

TFs, Transcription factors.