Novel assay to detect increased level of neutralizing anti-interferon gamma autoantibodies in non-tuberculouse mycobacterial patients

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Abstract:

Subjects exposed to non-tuberculouse mycobacterium (NTM) species do not always develop an active disease, which likely reflects underlying host susceptibility factors. Recent reports have shown that anti interferon gamma (IFN-γ) neutralizing autoantibodies (IFN-γ Ab) are associated with the development of disseminated NTM in patients without known evidence of immunodeficiency. The purpose of this study is to establish the screening method if subjects have IFN-γ Ab. Whole blood was obtained from patients with disseminated NTM, those with pulmonary NTM, and healthy controls. The neutralizing capacity to IFN-γ activity was assessed as an inhibition of Signal Transducer and Activation of Transcription 1 (STAT-1) phosphorylation in leukocyte after stimulation with exogenous IFN-γ by flow cytometer. The strength of phosphorylation was described as STAT1 phosphorylation index. Antigen capture assay was performed to measure the relative titer of Immunoglobulin-G fraction of IFN-γ Ab. STAT1 phosphorylation by IFN-γ was significantly inhibited in the leukocytes from patients with disseminated NTM compared to that in

healthy subjects, while this inhibition was not observed in patients with pulmonary NTM. All subjects with inhibited STAT1 phosphorylation had high titer of Immunoglobulin-G that reacted with IFN-γ in the antigen capture assay. The measurement of STAT1 phosphorylation index in whole blood leukocytes and antigen capture assay are simple and useful method for detection of anti- IFN-γ neutralizing autoantibodies, and is valuable in the pathophysiological diagnosis of disseminated NTM patients without obvious immunodeficiency.

Introduction

Although non-tuberculous mycobacteria (NTM) are ubiquitous in the environment, subjects exposed to NTM do not always develop active infectious disease, suggesting that underlying host susceptibility factors are important. Increased susceptibility to intracellular pathogens including NTM and salmonella, leading to severe infection is often caused by genetic defects that impair Interferon-gamma (IFN-γ) and Interleukin-12 (IL-12) mediated immunity [1]. Severe and progressive disease state could be developed in these patients despite prolonged antibiotic therapy. Recent reports have shown that neutralizing anti IFN-γ autoantibodies (IFN-γ Ab) are associated with disseminated NTM patients without known evidence of immunodeficiency, and that patients with IFN-γ Ab have impaired IFN-γ signaling leading to severe disseminated infections by intracellular pathogens, especially by NTM [2-6].

IFN-γ controls some of important gene expressions through the intracellular signaling via its own cell-surface receptor [7]. In this well characterized signal transduction pathway, ligand-receptor binding activates a member of the Janus

kinase family associated with an intracellular domain of the receptor and Signal Transducer and Activation of Transcription 1 (STAT-1) dimerization by its phosphorylation. Phosphorylated STAT1 translocate into nucleus and control gene expressions as a transcription factor.

Detection of autoantibodies against IFN-γ has also been shown in healthy individuals [8]. However, it is not yet clear whether they have biologically neutralizing capacity or not even in the healthy subjects. It can be assumed both presence of IFN-γ Ab and its neutralizing capacity are necessary for the onset of disseminated NTM infections in theory.

Here, in order to assess the underlying pathophysiology of disseminated NTM, we report two of novel assay to evaluate the presence of IFN-γ Ab and its neutralizing capacity. We quantified relative titer of Immunoglobulin-G (Ig-G) which recognizes IFN-γ in subjects' plasma or sera by antigen capture assay. The other bioassay evaluated IFN-γ signaling in human whole blood leukocyte based on IFN-γ stimulated, IFN-γ receptor-mediated phosphorylation of STAT1, which is named the STAT1 phosphorylation index (STAT1-PI).

Methods

Participants

This study was performed with the approval of the Ethics Committees at the School of Medicine, Niigata University（Approval number; 1413）. Written informed consent was obtained from all participants. All subjects had no congenital and acquired immune deficiency episodes such as HIV infection, Cancer and history of immunosuppressive therapy. Pulmonary non-tuberculous mycobacterial disease (pNTM) was diagnosed by following the diagnostic guidelines of The Japanese Respiratory Society and The Japanese Society for Tuberculosis society [9]. Disseminated NTM (dNTM) disease was defined by the existence of extra-pulmonary lesions, which was evident by the direct detection by culture of NTM species from them. Four cases of dNTM, 5 cases of pNTM and 7 healthy subjects were recruited. Mycobacterium avium complex (MAC) alone was cultured in 3 dNTM and all of 5 pNTM patients. One patient with dNTM was also detected Mycobacterium gordonae in addition to MAC.

Detection of STAT1 phosphorylation in leukocyte

113 Forty five μl of Heparinized whole blood, which was drawn from participants, was
114 transferred into polystyrene Round-Bottom Tube. Five μl of various
115 concentrations of recombinant human IFN-γ (rhIFN-γ) (Wako Co. Ltd., Gunma,
116 Japan) was added and subsequently incubated at 37℃ for indicated time. Red
117 blood cells were lysed and leukocytes were fixed. (Phosflow Lyse/Fix Buffer, BD
118 Bioscience) After washing, cells were permeabilized with 95% methanol on ice
119 for an hour. Permeabilized leukocytes were incubated with 20 μl of anti-mouse
120 phospho-STAT1 antibody (Alexa Fluor 647 Mouse Anti-STAT1 (pY701), BD
121 Biosciences). Positive cells with phosphorylated STAT1 were evaluated using
122 FACS callibure (BD Bioscience) and analyzed using CellQuest Pro software (BD
123 Bioscience). The STAT1-PI was calculated as the mean fluorescence of cells
124 primed with assigned concentration of IFN-γ minus that of unprimed cells divided
125 by that of unprimed cells, and then multiplied by 100.

126 **Interferon γ inhibition assay**

127 Effect of exogenous IFN-γ-Ab to STAT1-PI was examined. Various
128 concentrations of monoclonal goat anti-human IFN-γ neutralizing antibodies

(R&D systems, Inc., Minneapolis, USA), which consist of Ig-G class, or control Ig-G (R&D systems, Inc., Minneapolis, USA) was added to whole blood from healthy control. Leukocytes were stimulated with 50ng/ml of IFN-γ and their STAT1-PI was measured.

Immunoblot Assay

Ig-G was purified from 3 ml of patients' plasma using protein G coupled affinity chromatography column (Thermo Fisher Scientific, Rockford, IL, USA) following manufacture's protocol. Two hundreds of rhIFN-γ was subjected to SDS-PAGE under reducing condition, transferred electrophoretically to a PVDF membrane, and the membrane was incubated with 50 μg/ml of purified Ig-G for an hour after being treated with a blocking reagent. To detect human Ig-G, the membrane was incubated with peroxidase-labeled anti-human Ig-G antibody overnight at 4℃. Color was developed using Pierce Western Blotting Substrate Plus (Thermo Fisher Scientific, Rockford, IL, USA).

Antigen Capture Assay and relative quantification of IFN-γ-Ab titer

Plasma was diluted 1000-fold with 10% bovine serum albumin (BSA)

containing phosphate-buffered saline (PBS). A volume of 50 µl of diluted sera was transferred to a 96 well Coaster® EIA/RIA Plate (Corning Inc. NY, USA) coated with 1 μg/ml of rhIFN-γ and the plate was kept at room temperature. After washing, autoantibodies captured by rhIFN-γ were detected by peroxidase-labeled anti-human Ig-G antibody (Life Technologies Corporation, Carlsbad, CA USA) diluted 3000-fold with 10% BSA containing PBS. After washing, color was developed using tetramethylbenzidine and the absorbance was measured at 450 nm.

Statistical Analysis

The comparison of data among three groups was performed using Mann-Whitney U test. P-value below less than 0.05 was considered to be significant.

Result

STAT1 phosphorylation by rhIFN-γ in whole blood leukocyte

When whole blood leukocytes from healthy subjects were stimulated with 0 - 100 ng/ml of IFN-γ for 15 minutes, proportion of phosphorylated-STAT1 (pSTAT1) positive cells were increased in a dose dependent manner (Figure 1a). To standardize the proportion of pSTAT1, we evaluated STAT1-PI as described in the method section. The STAT1-PI increased in proportion to IFN-γ concentration reaching a maximum value and plateau phase between 50 to 200 ng/ml of IFN-γ stimulation (Figure 1b). When stimulated with 100 ng/ml of IFN-γ, the index was increased immediately and reached maximum at the point of 20 minutes after the stimulation, then gradually decreased (Figure 1c). Therefore, A 100 ng/ml of IFN-γ concentration and 15 minutes of stimulation time were determined as the standard stimulating dose and time respectively.

The effect of exogenous anti-IFN-γ neutralizing antibodies and evaluation of neutralizing capacity in whole blood from disseminated NTM subjects

177 STAT1-PI was clearly suppressed by exogenous anti-IFN-γ polyclonal
178 neutralizing antibodies in a concentration-dependent manner (Figure 2).
179 In healthy subjects, exogenous IFN-γ increased STAT1-PI in a dose
180 dependent manner. On the other hand, this finding was completely diminished in
181 disseminated NTM subjects (Figure 3a). Next we quantified STAT1-PI in order to
182 evaluate the presence or absence of neutralizing capacity against IFN-γ in
183 patients with dNTN, pNTM, and Healthy Subjects (Figure 3b). STAT1-PI of whole
184 blood leukocyte from dNTM patients was absolutely inhibited, while the inhibition
185 was not observed in those from pNTM and healthy subjects (n=4: -0.4 ± 13.2,
186 n=5: 78.6 ± 29.1, n=7: 114.2 ± 79.2). STAT1-PI was attenuated over time even in
187 healthy subjects; however, determination of the presence of neutralizing activity
188 was detectable even 3 days after phlebotomy in this method. (Figure 3c)

189 **Autoantibodies to IFN-γ in plasma of dNTM patients**

190 By the immunoblotting assay, Ig-G antibody specifically bound to rhIFN-γ was
191 detected in dNTM, but not in healthy subject (Figure 4a, b). Purified Ig-G from
192 normal subjects had little IFN-γ binding activity. On the other hand, Ig-G from

193 disseminated NTM subjects had high binding activity to IFN-γ. Furthermore, all
194 of disseminated NTM subjects with inhibited STAT1-PI have high titer of
195 IFN-γ-Ab confirmed by antigen capture assay, although we could not detect the
196 increased titer of IFN-γ-Ab among pulmonary NTM and healthy subjects (Figure
197 4c). These findings allow us to conclude that all of disseminated NTM subjects
198 have anti IFN-γ neutralizing autoantibodies.
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Discussion

The prevalence of the NTM infectious disease is increasing in a past decade, and most are chronic progressive respiratory infectious diseases, but there is often much difficulty to control of the disease condition by the conventional chemotherapy of the multiple drug combination. The biological characteristic of the person with high susceptibility is not clear; therefore the establishment of an effective intervention method should be hard way to reach. In considering the patient background in the infectious disease, the reports clarified the factor of the host side with high susceptibility to dissemination NTM infectious disease in recent years is very attractive [5]. For such the disease group, not only development of antibacterial chemotherapy, but also of the intervention for the factor of the host side may be necessary.

To date the method for the detection of the anti-IFN-γ neutralizing autoantibody existence in the disseminated NTM patients without obvious immunodeficiency has not been established. Although, among young patient, genetic search about genes, which participate in IFN-γ – IL-12 axis, should be

225 considered, it might not give us useful information for diagnosis, because the
226 patients with disseminated NTM with IFN-γ-Ab tend to be middle to high aged [1,
227 10-13]. The pathophysiological diagnosis and its interpretation in this disease
228 are often very difficult in clinical setting.

229 In addition to the difficulties on diagnosis, the intractability is also underlying
230 on treatment strategy. Some cases with anti IFN-γ-Ab have been reported to be
231 required long-term conventional chemotherapy for recovery from critical
232 conditions [4] [6]. Furthermore the recurrences are often reported and two died
233 case have been reported [14, 15]. The latest report suggested that this disease
234 should be recognized as a concept of the autoimmune disease. As this rationale,
235 anti-CD20 antibody (rituximab) was administrated for the purpose of controlling
236 an antibody-producing cells, which lead to a clinical improvement had been
237 reported [16]. There might be the possibility that can produce the big paradigm
238 shift of the strategy for the treatment for this infectious disease. On such a point
239 of view, it is important for patient profit to get definite diagnosis in early stage of
240 the disease.

241 Our newly developed methods have made clear the existence of the IFN-γ-Ab
242 by performing both measurement of STAT1-PI, which is examined neutralizing
243 capacity to IFN-γ and the antigen capture assay. Both of them are necessary for
244 diagnosis, because, in either one, the existence of the soluble receptor and the
245 non-neutralization autoantibody were possible. The limitation of our study was
246 that we could not determine the specificity and sensitivity of these methods
247 because of the insufficient numbers of participants. In this study, we could prove
248 the existence of the IFN-γ-Ab in all four dNTM subjects without the apparent
249 immunodeficiency. On the other hand, the IFN-γ-Ab was not detected in all of
250 pNTM and healthy subjects. Therefore, usefulness of these two methods for
251 detection of IFN-γ-Ab in clinical setting might be determined, although we need
252 to evaluate more numbers of subjects.

253 The STAT1-PI with whole blood leukocytes and the antigen capture assay are
254 simple and useful method for detection of anti-IFN-γ neutralizing autoantibodies
255 in the evaluation of disseminated NTM patients without obvious
256 immunodeficiency. In the practical clinical setting, these methods should help us

257 to get a definite pathophysiological diagnosis.

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264

265 **Conflict of interest**

266 None.

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Figure legend

Figure 1. Evaluation of IFN-γ signaling in a healthy subject.

a. STAT1 phosphorylation in leukocyte was detected by Flow cytometer. Myeloid cells were gated on dot plot for further analysis. IFN-γ increased proportion of phosphorylated STAT1 positive cells in a dose dependent manner. **b.** STAT1-PI was increased in dose dependent manner and reached maximum and plateau phase between 10-200ng/ml of IFN-γ stimulation. **c.** The index was increased immediately and reached maximum at the point of 20 minutes after IFN-γ stimulation and gradually decreased. Each experiment was done multiple times. Representative data are shown.

Figure 2. Effect of exogenous anti-IFN-γ neutralizing antibodies

Relative STAT1-PI, which was derived from ratio to STAT1-PI mixed with control Ig-G, was decreased in dose dependent manner of exogenous IFN-γ neutralizing antibodies and reached zero between 10-100μg/ml of IFN-γ Ab concentrations. Exogenous Ig-G did not affect STAT1-PI even if concentration was increasing. White circles indicate the data with neutralizing IFN-γ Ab, black

353 circles indicate that with control Ig-G. This experiment was done multiple times.
354 Representative data are shown.

355 **Figure 3. Evaluation of neutralizing capacity to IFN-γ by STAT-1**
356 **phosphorylation index**

357 **a.** STAT1-PI was not increased at all even with stimulation with 1000ng/ml of
358 IFN-γ in a dNTM patient with IFN-γ Ab. White and black circles indicate the data
359 from dNTM and a healthy subject respectively. This experiment was done
360 multiple times. Representative data are shown. **b.** STAT1-PI was nearly zero in
361 dNTM, when whole blood was stimulated with 10 ng/ml of IFN-γ. Each group
362 included 4, 5 and 7 subjects respectively. **c.** STAT1-PI was diminished in a day
363 dependent manner after phlebotomy. STAT1-PI was evaluated with whole blood
364 kept in 4 degree refrigerator till the time of measurement. White and black circles
365 indicate the data from disseminated NTM and a healthy subject respectively.
366 This experiment was done multiple times. Representative data are shown.

367 **Figure 4. Specific binding of purified IgG from dNTM to IFN-γ and**
368 **Quantification of relative titers of IFN-γ Ab in plasma by antigen capture**

assay.

a. rhIFN-γ was subjected to SDS-PAGE under reducing conditions. The proteins were stained with coomassie blue. **b.** After transferred to PVDF membrane incubated with purified Ig-G from disseminated NTM or Healthy subjects and confirmed specific binding. Only Ig-G from dNTM could bind to IFN-γ**. c.** Relative IFN-γ Ab concentrations in plasma to healthy subjects were quantified. Mean concentrations of disseminated NTM subjects was 14.7 folds (range; 5.4 – 28.6) and of pulmonary NTM subjects was 1.4 folds (range; 0.75 – 2.0) to healthy subjects ($p<0.01$ and $p=0.01$, respectively).

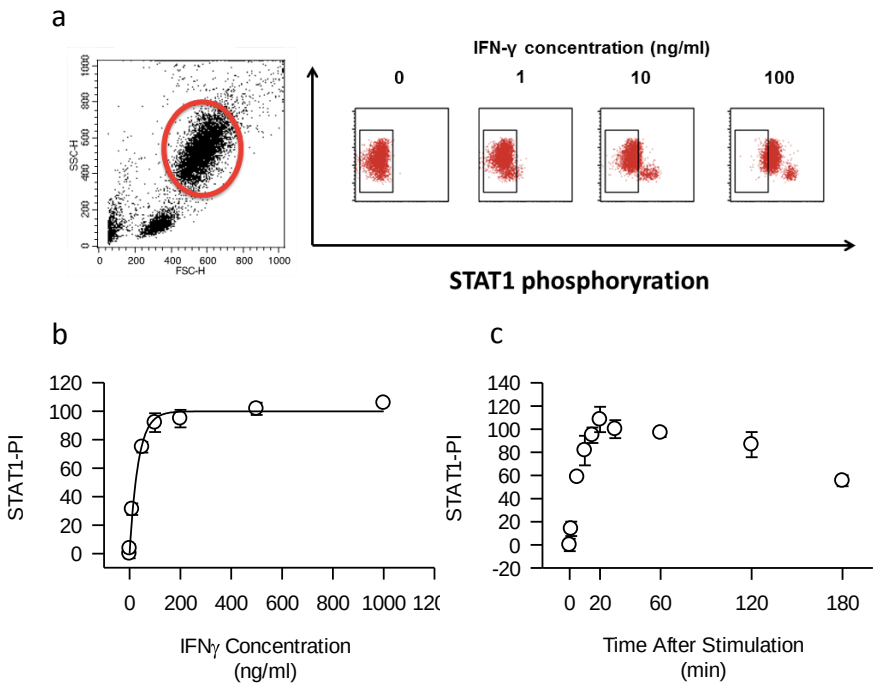

a
IFN-γ concentration (ng/ml)
0
1
10
100
SSC-H
FSC-H
STAT1 phosphoryration
b
STAT1-PI
IFNγ Concentration
(ng/ml)
c
STAT1-PI
Time After Stimulation
(min)

Figure 1

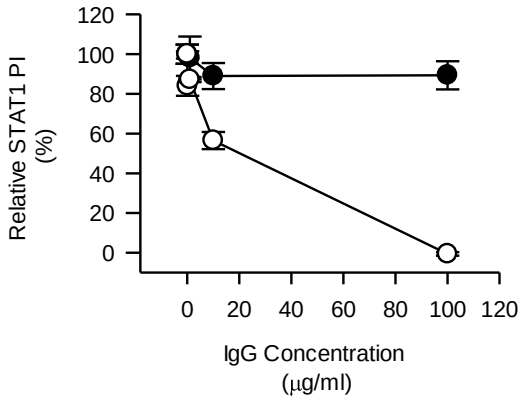
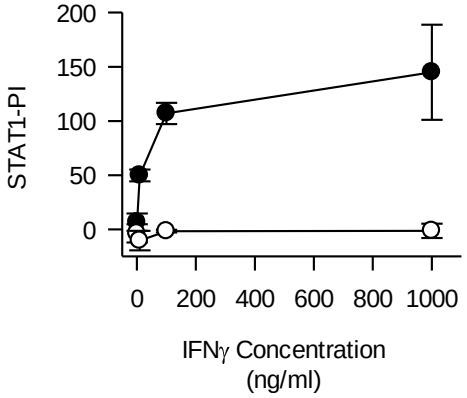
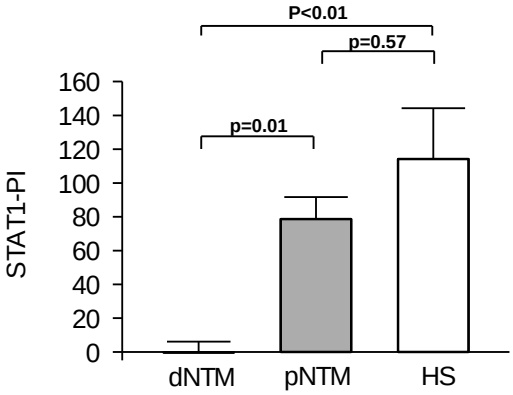

Relative STAT1 PI
(%)
120
100
80
60
40
20
0
0
20
40
60
80
100
120
IgG Concentration
(μg/ml)

Figure 2

a


200
150
100
50
0
STAT1-PI
0
200
400
600
800
1000
IFNγ Concentration
(ng/ml)

b


P<0.01
p=0.57
p=0.01
160
140
120
100
80
60
40
20
0
STAT1-PI
dNTM
pNTM
HS

c

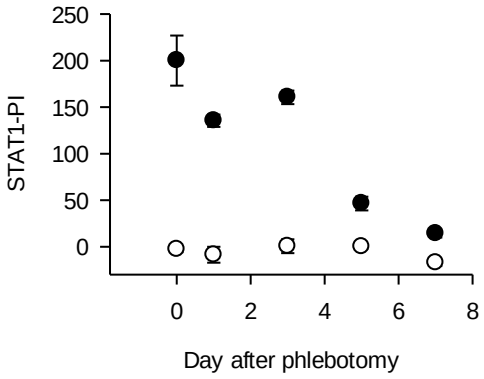

250
200
150
100
50
0
STAT1-PI
0
2
4
6
8
Day after phlebotomy

Figure 3

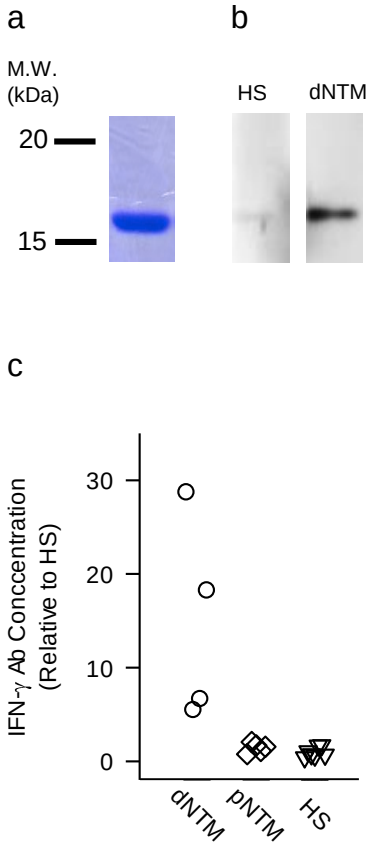

a
b
M.W.
(kDa)
20
15
HS
dNTM
c
IFN-γ Ab Conccentration
(Relative to HS)
30
20
10
0
dNTM
pNTM
HS

Figure 4