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Inter-rater agreement in the assessment of abnormal chest X-ray findings for tuberculosis between two Asian countries

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Abstract

Background: Inter-rater agreement in the interpretation of chest X-ray (CXR) films is crucial for clinical and epidemiological studies of tuberculosis. We compared the readings of CXR films used for a survey of tuberculosis between raters from two Asian countries.

Methods: Of the 11,624 people enrolled in a prevalence survey in Hanoi, Viet Nam, in 2003, we studied 258 individuals whose CXR films did not exclude the possibility of active tuberculosis. Follow-up films obtained from accessible individuals in 2006 were also analyzed. Two Japanese and two Vietnamese raters read the CXR films based on a coding system proposed by Den Boon et al. and another system newly developed in this study. Inter-rater agreement was evaluated by kappa statistics. Marginal homogeneity was evaluated by the generalized estimating equation (GEE).

Results: CXR findings suspected of tuberculosis differed between the four raters. The frequencies of infiltrates and fibrosis/scarring detected on the films significantly differed between the raters from the two countries ($P < 0.0001$ and $P = 0.0082$, respectively, by GEE). The definition of findings such as primary cavity, used in the coding systems also affected the degree of agreement.

Conclusions: CXR findings were inconsistent between the raters with different backgrounds. High inter-rater agreement is a component necessary for an optimal CXR coding system, particularly in international studies. An analysis of reading results and a thorough discussion to achieve a consensus would be necessary to achieve further consistency and high quality of reading.

Background

Despite its several disadvantages, chest radiography remains an important supporting tool in tuberculosis (TB) surveys and clinical management of active disease [1-3]. Chest X-ray (CXR) findings should be carefully assessed because of its potential problems such as low specificity and insufficient reproducibility [4].

In this context, reading methods that are less influenced by raters are required and several CXR coding systems have been proposed [5-7]. In general, complex interpretation codes hamper intra- and inter-rater agreement and simple codes are preferred [6,7], because reproducible and validated coding system may be useful in monitoring disease in clinical and epidemiological studies [8,9].

Previous studies suggest that variability in CXR interpretation among raters is attributed to subjective reading accompanied by insufficient experience or different professional background of the raters [7,10-12]. However, the relationship between agreement levels and relevant factors that may cause disagreement, particularly influence of medical background including different national origins has not been characterized.

In the present study, Vietnamese and Japanese raters studied the readings of suspected TB lesions on CXR films taken during a survey of TB prevalence in Hanoi,

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Viet Nam [3]. The follow-up films were also compared with the initial films. As analytical tools, two different types of coding systems were used: One was previously reported by another group [5] and the other was newly developed in this study. The aim of the study was to highlight inter-rater agreement between raters with different medical backgrounds. We also attempted to characterize the optimal codes or coding systems used in international studies for a simple and objective evaluation of CXR findings suspected of TB.

Methods

Ethics approval

This study was approved by the ethics committees of the Ministry of Health, Viet Nam and the National Center for Global Health and Medicine (formerly, International Medical Center of Japan). Written informed consent was obtained from each participant prior to the investigations, including the prevalence survey and the follow-up study.

Study population

A population-based TB prevalence survey of 11,624 people aged 15 and over was conducted in Hanoi in 2003 as reported previously [3]. Briefly, subjects suspected of having active TB based on CXR or on symptoms underwent sputum smear microscopy and/or mycobacterial culture. Details of HIV status were not obtained from the study subjects. According to the report of World Health Organization during this period, estimated prevalence of HIV co-infection in new TB patients aged 15-49 was relatively low (2.8%) in Viet Nam [13].

Barring 317 individuals, active TB was radiographically excluded for the rest. Of these 317 individuals, 22 (6.9%) were diagnosed by bacteriological methods, including sputum culture [3]. In 2004, individuals who presented with radiographic findings during the initial survey were advised to undergo sputum smear and culture tests following the World Health Organization recommendation [14,15]. In the 2006 follow-up, in which the same group of individuals was recalled for plain chest radiographic examination (AGFA X-ray film, Beijing, China; Shimadzu UD 150L-30V, Kyoto, Japan) and sputum test, including direct smear and culture. Using a questionnaire, we collected information regarding individual history, additional examinations performed, and treatment for TB undergone after the initial survey. Demographic information (including addresses) collected during the prevalence survey was used to trace the target group in the follow-up period.

The CXR films analyzed in this study were those in which active TB had not been radiographically excluded during the prevalence survey and were those taken during the follow-up in 2006. In total, 258 of the 317 films in the prevalence survey and 93 follow-up films were available at the time of analysis in this study. The rest of TB-suspected films in the prevalence survey were missing.

CXR coding systems and reading of films

Two coding systems were used to classify the CXR findings. The chest radiograph reading and recording system (CRRS) was developed in 2005 to detect TB and other forms of lung disease [5]. Profusion score and details of abnormalities unrelated to TB were omitted. All the other coding items of this system were retained. A Japan-Vietnam CXR coding system (JVCS) (Figure 1) consisting of rather simple codes was also used: We newly developed this system, considering a registration form used in a public payment system for TB treatment expenses in Japan and reading practice in Viet Nam. CRRS classifies parenchymal abnormalities as primary or secondary lesions depending on the significance of the lesion. In contrast, JVCS does not consider the significance of the lesion, though it records pleural effusion and thickening separately. Additionally, CRRS classifies nodules based on their size and calcification, whereas JVCS separately records nodules and calcification.

Two Japanese pulmonary physicians (E.T. and N.K.) and two Vietnamese radiologists (L.D.H. and P.T.C.) read the CXR films. These readers were different from those who read the CXR films during the initial survey. All CXR films were first read using CRRS. After the completion of readings by CRRS, CXR films were read using JVCS without the results of CRRS being made known to the readers. Each reader was also blinded to the others' readings and clinical information. Instruction and training regarding the two coding systems were given prior to the actual reading. The four raters were asked to reach a consensus while assessing 10 standard films from Japan and another 10 films from Viet Nam.

Statistical analysis

We adopted a double entry system of data entry. JMP version 7.0.1 (SAS Institute Inc., Cary, NC, USA) and SAS version 9.1 (SAS Institute Inc.) were used for analysis. Kappa statistics were used to investigate inter-rater agreement on the presence or absence of lesions of interest. We adopted the following guidelines for interpretation of kappa coefficients: < 0, poor agreement; 0-0.20, slight; 0.21-0.40, fair; 0.41-0.60, moderate; 0.61-0.80, good; and 0.81-1.00, very good [16-18]. Weighted kappa was used to assess inter-rater agreement on variables with more than two categories. McNemar's test or its extension, Bowker's test of symmetry, was used to investigate the symmetry of disagreement between two raters, which tests whether the frequency of an abnormality detected by one rater is significantly different from that by another rater. The generalized estimation equation (GEE) was also used to test the

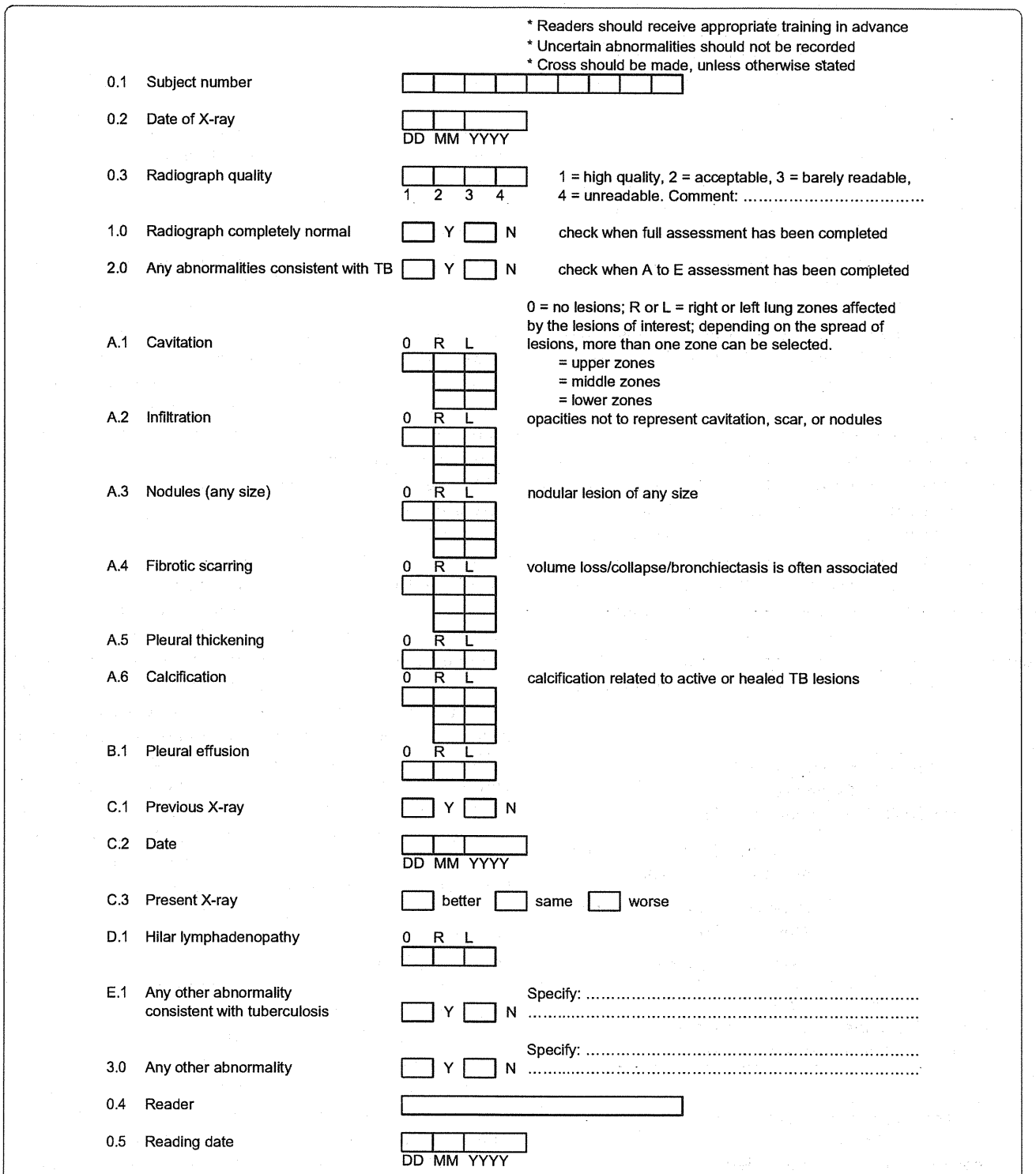

Figure 1 Chest X-ray coding: JVCS. JVCS = Japan-Vietnam chest X-ray coding system; DD = date in two digits; MM = month in two digits; YYYY = year in four digits; Y = yes; N = no; R = right; L = left.

similarities in frequencies of positive findings between groups of raters (marginal homogeneity). No symmetry or non-marginal homogeneity was considered to be significant when $P < 0.05$.

Results

Follow-up after TB prevalence survey

In 2004, one year after the prevalence survey, 204 (64.4%) of the 317 individuals who presented with

radiographic findings of suspected TB underwent a sputum smear test, one of whom tested positive. The initial CXR film of this case showed infiltrates, fibrosis/scarring, and calcification. The follow-up radiograph in 2006 showed improvement after treatment.

In the follow-up in 2006, 93 individuals were checked, one of whom was diagnosed by smear and culture as TB positive (Figure 2). Besides calcification, which was seen in the initial CXR film, infiltrates were present in the follow-up film. All raters evaluated this case as "worse" based on the radiographic findings.

In total, five individuals were reported to have active TB during the 3-year follow-up period. Two were diagnosed bacteriologically and three were diagnosed based on self-reported TB episodes. All the films were randomly mixed in the study set.

Inter-rater agreement on CXR findings

Using the two coding systems, four raters assessed the 258 films taken during the 2003 prevalence survey; two raters assessed the 93 films taken in the 2006 follow-up. A total of 2,436 readings were conducted (Figure 2).

Agreement levels regarding overall parenchymal abnormalities assessed by CRRS varied. Their kappa values were interpreted as fair to good, ranging from 0.24 to 0.63, from the following six comparisons: a comparison between the two Japanese raters (JP-JP); four comparisons between Japanese and Vietnamese raters (JP-VN (1) to (4)); and a comparison between the two Vietnamese raters (VN-VN) (Table 1). Agreement levels regarding calcification also varied. They were considered as fair to good with JVCS and slight to fair with CRRS. Kappa values for pleural effusion with JVCS were interpreted as moderate to good, ranging from 0.54 to 0.77, indicating high level of agreement irrespective of country or rater.

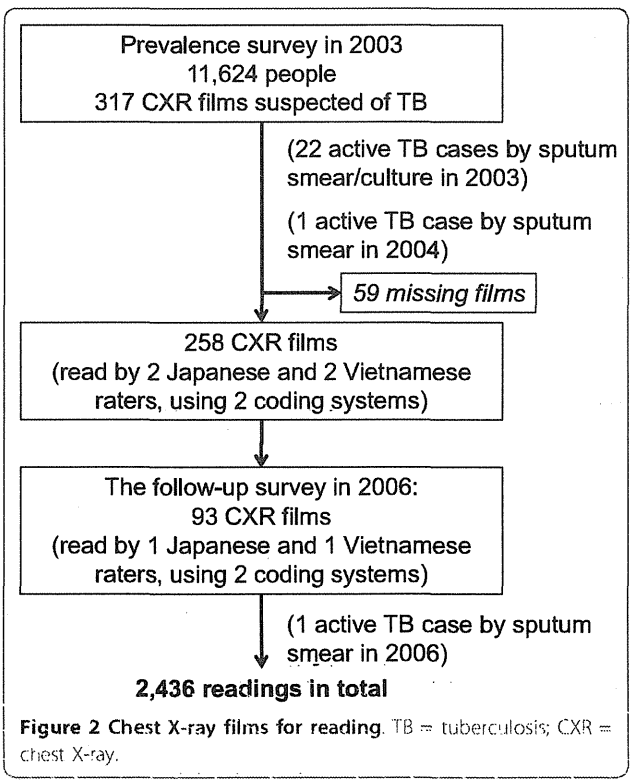

Figure 2 Chest X-ray films for reading. TB = tuberculosis; CXR = chest X-ray.

Major parenchymal findings, cavity, fibrosis/scarring, infiltrates, and nodules were assessed in a similar way, as shown in Table 2. Agreement levels regarding primary and secondary cavities in CRRS were rather low (kappa values ranged from -0.02 to 0.36) except for relatively high agreement levels regarding a primary cavity between the Japanese raters (kappa = 0.60), and a secondary cavity between the Vietnamese raters (kappa = 0.43). Cavitation was, thus, mainly classified as a primary lesion by the Japanese raters and as a secondary lesion by the Vietnamese raters.

Although agreement levels relating to fibrosis/scarring were also low, kappa values for secondary fibrosis/scarring with CRRS revealed fair levels of agreement between raters from the same country (kappa = 0.28 [JP-JP] and 0.22 [VN-VN]), but revealed only slight agreement between raters from different countries (kappa = 0.11 to 0.20 [JP-VN]). Among all Japanese-Vietnamese pairs, the Vietnamese raters specified secondary fibrosis/scars more frequently than the Japanese raters ($P = 0.0001$ or $P < 0.0001$ by McNemar test). The frequency of positive findings of secondary fibrosis with CRRS by both Vietnamese raters was 26/255 (10.2%), whereas that by both Japanese raters was only 7/245 (2.9%) (Table not shown). The frequency of positive findings of fibrosis/scarring with JVCS by both Vietnamese raters (56/255 = 22.0%) also tended to be higher than that by both Japanese raters (42/245 = 17.1%). GEE further confirmed the significant difference in frequencies of fibrosis/scarring between raters from different countries ($P = 0.0082$).

Agreement levels regarding infiltrates between the two raters from the same country were considered as moderate (kappa = 0.49 [JP-JP] and 0.57 [VN-VN]) and as fair between two raters from different countries (kappa = 0.21 to 0.30 [JP-VN]) according to JVCS (Table 2). The Japanese raters detected infiltrates more frequently than the Vietnamese raters ($P < 0.0001$ by McNemar test) in all comparisons. The frequency of positive findings of primary infiltrates with CRRS by both Japanese raters was 68/245 (27.8%), whereas that by both Vietnamese raters was only 22/255 (8.6%) (Table not shown). The frequency of positive findings of infiltrates with JVCS by both Japanese raters (119/245 = 48.6%) also tended to be higher than that by both Vietnamese raters (46/255 = 18.0%). The different frequencies of positive

Table 1 Inter-rater agreement with respect to general and parenchymal findings for each coding system (n = 258)

Item	Coding system	Inter-rater agreement					
		Kappa with 95% confidence interval and the absolute number of films (-/-+/+-/++)					
		JP-JP	JP-VN				VN-VN
			[1]	[2]	[3]	[4]	
Total number	JVCS	(245)	(246)	(245)	(245)	(244)	(255)
of tested films	CRRS	(245)	(245)	(246)	(246)	(245)	(255)
Parenchymal	JVCS	NA	NA	NA	NA	NA	NA
abnormality	CRRS	0.63 [0.51-0.75]	0.24 [0.16-0.32]	0.50 [0.38-0.62]	0.25 [0.16-0.34]	0.58 [0.46-0.70]	0.27 [0.17-0.37]
		(32/21/7/185)	(9/44/0/192)	(24/29/6/187)	(7/32/2/205)	(22/17/8/198)	(6/3/24/222)
Calcification*	JVCS	0.62 [0.49-0.75]	0.47 [0.35-0.59]	0.21 [0.12-0.30]	0.55 [0.42-0.68]	0.30 [0.21-0.39]	0.26 [0.17-0.35]
		(188/13/14/30)	(187/14/22/23)	(198/2/38/7)	(190/12/18/25)	(201/0/34/9)	(215/2/31/7)
	CRRS	0.28 [0.15-0.41]	0.35 [0.23-0.47]	0.15 [0.04-0.26]	0.36 [0.25-0.47]	0.36 [0.25-0.47]	0.17 [0.09-0.25]
		(219/10/11/5)	(206/23/6/10)	(226/4/14/2)	(208/23/5/10)	(228/2/11/4)	(219/2/30/4)
Pleural	JVCS	0.58 [0.45-0.71]	0.77 [0.64-0.90]	0.66 [0.54-0.78]	0.64 [0.51-0.77]	0.54 [0.41-0.67]	0.73 [0.61-0.85]
effusion		(222/8/5/10)	(226/5/2/13)	(222/8/3/12)	(221/6/6/12)	(217/9/7/11)	(230/6/4/15)
Pleural	JVCS	0.35 [0.23-0.47]	0.22 [0.14-0.30]	0.45 [0.33-0.57]	0.28 [0.18-0.38]	0.45 [0.32-0.58]	0.31 [0.22-0.40]
thickening		(158/39/19/29)	(88/110/2/46)	(161/36/14/34)	(84/93/6/62)	(148/28/26/42)	(87/4/95/69)
Pleural	CRRS	0.30 [0.20-0.40]	0.16 [0.09-0.23]	0.46 [0.34-0.58]	0.45 [0.33-0.57]	0.54 [0.42-0.66]	0.32 [0.22-0.42]
abnormalities		(130/73/6/36)	(83/120/3/39)	(159/44/7/36)	(76/60/10/100)	(124/12/42/67)	(81/6/91/77)
Pleural effusion/	CRRS	0.48 [0.36-0.60]	0.34 [0.22-0.46]	0.55 [0.43-0.67]	0.49 [0.36-0.62]	0.67 [0.55-0.79]	0.48 [0.36-0.60]
thickening**		(176/35/6/28)	(168/43/11/23)	(189/22/9/26)	(156/26/23/41)	(176/6/22/41)	(173/14/33/35)

* In CRRS, calcification here indicates calcified granuloma only

**In CRRS, pleural effusion and thickening are combined

JVCS Japan-Vietnam chest X-ray coding system, *CRRS* chest radiograph reading and recording system, *TB* tuberculosis, *NA* not applicable, *JP-JP* a comparison between the Japanese raters; *JP-VN* [1] to [4] comparisons between Japanese-Vietnamese raters, *VN-VN* a comparison between the Vietnamese raters, (-/-+/+-/++) (negative findings by both raters/positive findings only by the second rater/positive findings only by the first rater/positive findings by both raters)

infiltrate readings between the raters from the two countries were also confirmed by using GEE ($P < 0.0001$).

The levels of inter-rater agreement were considered slight to fair for nodules, irrespective of the raters' home country or the coding system used.

Table 2 Inter-rater agreement with respect to parenchymal findings for each coding system (n = 258)

Item	Coding system	Inter-rater agreement					
		Kappa with 95% confidence interval					
		JP-JP	JP-VN				VN-VN
			[1]	[2]	[3]	[4]	
Total number	JVCS	(245)	(246)	(245)	(245)	(244)	(255)
of tested films	CRRS	(245)	(245)	(246)	(246)	(245)	(255)
Cavity	JVCS	0.44 [0.32-0.56]	0.36 [0.25-0.47]	0.47 [0.34-0.64]	0.30 [0.20-0.40]	0.50 [0.38-0.62]	0.52 [0.40-0.64]
	CRRS primary *	0.60 [0.48-0.72]	0.10 [0.03-0.17]	0.28 [0.18-0.38]	0.06 [-0.02-0.14]	0.36 [0.25-0.47]	0.15 [0.04-0.26]
	CRRS secondary	-0.02 [-0.14-0.10]	0.04 [0.00-0.08]	0.06 [0.01-0.11]	0.00 [-0.05-0.05]	0.04 [-0.03-0.11]	0.43 [0.32-0.54]
Fibrosis/scar	JVCS	0.30 [0.17-0.43]	0.19 [0.07-0.31]	0.34 [0.22-0.46]	0.18 [0.05-0.31]	0.34 [0.23-0.45]	0.31 [0.20-0.42]
	CRRS primary	0.31 [0.18-0.44]	0.02 [-0.02-0.06]	0.27 [0.14-0.40]	-0.02 [-0.07-0.03]	0.15 [0.02-0.28]	0.03 [-0.01-0.07]
	CRRS secondary	0.28 [0.16-0.40]	0.20 [0.10-0.30]	0.11 [0.02-0.20]	0.14 [0.03-0.25]	0.16 [0.06-0.26]	0.22 [0.10-0.34]
Infiltrate	JVCS	0.49 [0.37-0.61]	0.30 [0.20-0.40]	0.27 [0.18-0.36]	0.22 [0.13-0.31]	0.21 [0.13-0.29]	0.57 [0.45-0.69]
	CRRS primary	0.33 [0.21-0.45]	0.24 [0.15-0.33]	0.31 [0.19-0.43]	0.15 [0.08-0.22]	0.22 [0.12-0.32]	0.41 [0.30-0.52]
	CRRS secondary	-0.05 [-0.18-0.08]	0.13 [0.03-0.23]	-0.02 [-0.12-0.08]	-0.04 [-0.14-0.06]	-0.02 [-0.12-0.08]	0.02 [-0.03-0.07]
Nodule	JVCS	0.27 [0.14-0.40]	0.11 [0.05-0.17]	0.26 [0.14-0.38]	0.09 [0.03-0.15]	0.31 [0.20-0.42]	0.19 [0.11-0.27]
	CRRS primary	0.37 [0.25-0.49]	0.13 [0.06-0.20]	0.40 [0.28-0.52]	0.09 [0.03-0.15]	0.24 [0.12-0.36]	0.21 [0.13-0.29]
	CRRS secondary	0.22 [0.10-0.34]	0.22 [0.11-0.33]	0.14 [0.02-0.26]	0.13 [0.03-0.23]	0.29 [0.16-0.42]	0.22 [0.12-0.32]

* Primary and secondary lesions are described in CRRS

JVCS Japan-Vietnam chest X-ray coding system, *CRRS* chest radiograph reading and recording system, *TB* tuberculosis, *NA* not applicable, *JP-JP* a comparison between the Japanese raters, *JP-VN* [1] to [4] comparisons between Japanese-Vietnamese raters, *VN-VN* a comparison between the Vietnamese raters

An overall assessment of CXR changes after 3 years was conducted by one of the two raters from each country. Agreement was moderate for both coding systems (weighted kappa = 0.47 and 0.40). The Japanese rater indicated deterioration more frequently than the Vietnamese rater (Table 3); this difference was considered highly significant for both JVCS and CRRS by the symmetry test (P = 0.0002 and 0.0008, respectively, by Bowker's test). When assessing changes in specific findings after 3 years, the Japanese rater detected infiltrates more frequently than the Vietnamese rater ($P < 0.0001$; Figure 3). Among 55 cases of infiltrates, 12 (22%) were assessed as "further spread" by the Japanese rater while 2 (8%) out of 25 cases of infiltrates were assessed as "further spread" by the Vietnamese rater (data not shown).

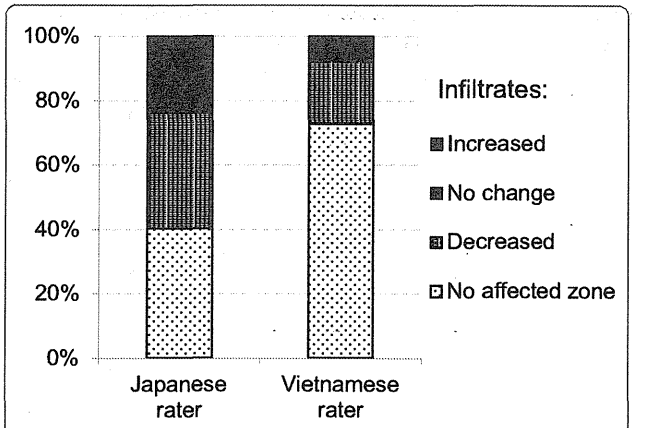

Figure 3 Infiltrates on chest X-rays after a 3-year interval. Evaluation of infiltrates on chest X-ray films between 2003 and 2006 using JVCS codes shows changes in the number of affected areas (upper, middle, and lower zones of each side of the lung). "No affected zone" indicates that the rater did not indicate the presence of infiltrates in either film. The Japanese rater detected infiltrates more commonly than did the Vietnamese rater ($P < 0.0001$), which corresponds with the results of a greater proportion of "increased" and a smaller proportion of "no detected zone" readings after 3 years. JVCS = Japan-Vietnam chest X-ray coding system

Discussion

Our study confirmed that the readings of CXR findings of suspected TB vary significantly among the raters. Differences in the backgrounds of the raters and different coding systems were considered potential factors affecting the levels of agreement. We found the following two patterns of marked tendency toward inconsistency in the CXR findings: 1) disagreement presumably attributed to the raters' home country and typically observed for infiltrates and secondary fibrosis/scarring and 2) disagreement observed for nodules, irrespective of the rater background. Through discussions conducted with the four raters after the trial, we identified some possible causes of this disagreement, though pre-existing problems were not disclosed when the standard films were checked prior to commencement of the study.

First, it is likely that this disagreement was partly caused by differences between countries regarding the definition of pulmonary lesions. For example, the Vietnamese raters limited the definition of infiltrates to relatively homogenous opacities greater than 10 mm in size, whereas the Japanese raters also included groups of smaller-sized scattered lesions with unclear margins in this classification. As a result, positive findings of infiltrates were more frequently reported by the Japanese raters.

Second, spontaneously cured mild TB resulting in parenchymal fibrosis or scarring, which is commonly seen in countries with high prevalence of TB, is a probable reason for the more frequent detection of these lesions by the Vietnamese raters. In addition, CT scans are compared with plain CXRs more commonly in Japan than in Viet Nam. This practice in TB diagnosis and management might affect the interpretations of the Japanese raters.

Disagreement between the raters from the two Asian countries could be attributed to many background factors, including the medical educational systems and on-the-job training imparted after graduation. In Japan, plain CXR films are read predominantly by clinicians, while in Viet Nam, radiologists also perform this role. Such differences are likely to affect the reading and should be taken into consideration in international studies. Even within a single country, inter-rater agreement depends on the experience of the raters [7,10,12] and is relatively low between raters in different centers [10].

Table 3 Overall assessment of radiographic findings after 3 years

JVCS					CRRS				
JP	VN			Total	JP	VN			Total
	Better	Same	Worse			Better	Same	Worse	
Better	23	6	0	29	Better	28	6	0	34
Same	18	21	0	39	Same	16	18	1	35
Worse	7	7	7	21	Worse	4	10	5	19
Total	48	34	7	89	Total	48	34	6	88
	Weighted kappa = 0.40 [0.22-0.57]					Weighted kappa = 0.47 [0.31-0.63]			

JVCS Japan-Vietnam chest X-ray coding system, *CRRS* chest radiograph reading and recording system, *JP* Japanese rater, *VN* Vietnamese rater

The tested coding systems had both advantages and disadvantages in the context of our study. With CRRS, parenchymal abnormalities are classified into primary and secondary lesions, and it is not easy for raters to differentiate between the two. The Japanese raters emphasized on cavitation and presence of infiltrates as primary lesions of active TB, but the Vietnamese raters objectively judged the primary lesions on the basis of the size of lesions and proportion of the lung involved.

Although fairly reproducible, a disadvantage of JVCS is that it cannot provide any information regarding the significance of active lesions. Thus, CRRS is more informative. Activity, however, is a subjective term and the reproducibility of this description apparently worsens when included in a coding system. This implies the limitations of the plain CXR as a classic imaging tool. It may be assumed that defining necessary medical terms carefully through training and in-depth discussion prior to actual reading would minimize misunderstandings, even with a detailed coding system. However, this was not effective in our study, possibly because of language barriers, different medical backgrounds, and insufficient recognition of the problems. Collectively, our results support the concept of reproducibility of a simplified coding system [6,7,19], which may be critical when a system is shared by raters from different countries, such as even Asian countries.

On comparing CXR findings 3 years after the prevalence survey, Japanese raters detected deterioration in more cases than Vietnamese raters. The fact that the Japanese raters more frequently detected infiltrates may partly explain this discrepancy, because infiltrates generally signify active lesions, though unknown factors may also have affected their readings. This should be considered when CXRs are used for follow-up because the radiological appearance of lesions will not provide sufficient information for monitoring TB unless patient history and bacteriological examination are combined [8,10,19].

Our study has several limitations. First, caution should be exercised when extrapolating the results to describe the way CXRs are generally read in the two Asian countries. Although different medical backgrounds in the countries were obvious after reviewing and discussing the results, the raters' qualifications should also be considered. Second, in the present study, the overall sensitivity and specificity of CXR-based diagnosis of tuberculosis were not determined because the number of active TB cases detected in our cohort study was rather small (< 10%) and because these parameters would be influenced more by individual raters' skills and experiences than by the coding system used. Third, the coverage rate of the radiographic follow-up study after 3 years was not high, one of the reasons being the rapid speed of urbanization and an increasingly mobile population in Hanoi, which caused difficulties when tracing particular individuals. Nevertheless, our findings present an important point to be considered in international studies of TB using a CXR coding system.

Conclusions

In our study, CXR findings of suspected TB were inconsistent between raters with different backgrounds, presumably because of differences in medical practice and education between the two countries. Although each coding system has its advantages and disadvantages, a simplified classification system is suitable for maintaining sufficient agreement between raters from different countries. To improve the quality of future international collaborative studies, harmony could be obtained between raters of different nationalities by thorough discussion regarding the possible causes of disagreement in CXR readings, using standard films and descriptions of major findings.

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Authors' contributions

SS and NTLH participated in supervising the on-site implementation of the study, drafting the paper, and substantially revising it. ET, LDH, PTC, and NKo read the chest X-ray films. LTL and PHT participated in the conception, design, and supervision of the study. PTNB participated in on-site implementation of the study. NI supervised and performed statistical analysis. NKe participated in the conception and design of the study, analysis and interpretation of data, drafting of the paper, and substantially revising it. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Critical role of AIM2 in *Mycobacterium tuberculosis* infection

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Abstract

Absent in melanoma 2 (AIM2) is a sensor of cytosolic DNA that is responsible for activation of the inflammasome and host immune responses to DNA viruses and intracellular bacteria. However, the role of AIM2 in host defenses against *Mycobacterium tuberculosis* is unknown. Here, we show that AIM2-deficient mice were highly susceptible to intratracheal infection with *M. tuberculosis* and that this was associated with defective IL-1β and IL-18 production together with impaired T_h1 responses. Macrophages from AIM2-deficient mice infected with *M. tuberculosis* showed severely impaired secretion of IL-1β and IL-18 as well as activation of the inflammasome, determined by caspase-1 cleavage. Genomic DNA extracted from *M. tuberculosis* (Mtb DNA) induced caspase-1 activation and IL-1β/IL-18 secretion in an AIM2-dependent manner. Mtb DNA, which was present in the cytosol, co-localized with AIM2. Taken together, these findings demonstrate that AIM2 plays an important role in *M. tuberculosis* infection through the recognition of Mtb DNA.

Keywords: host defense, inflammasome, macrophages

Introduction

Tuberculosis is caused by *Mycobacterium tuberculosis* and is a serious disease worldwide causing about 2 million deaths each year. The risk of disease is increased by the emergence of acquired immune deficiency syndrome and multidrug-resistant mycobacteria (1). *Mycobacterium tuberculosis* mainly invades and parasitizes macrophages by inhibiting phagosome maturation into phagolysosomes. Macrophages have several recognition systems to defend against mycobacterial invasion. Toll-like receptors (TLRs) recognize mycobacterial components such as glycolipids and CpG motif DNA (2–4). Several recent findings have also indicated that pattern recognition receptors other than TLRs, such as C-type lectin receptors and NOD-like receptors (NLRs), are implicated in the innate recognition of mycobacterial components (5–10). TLRs and C-type lectin receptors are membrane-bound molecules recognizing mycobacterial components in the extracellular compartments, whereas NLRs are present in the cytosol. Thus, several pattern recognition receptors showing distinct subcellular expression patterns recognize structurally and functionally different components of mycobacteria, contributing to protection by evoking an immune response.

Among the NLR family of proteins, NLR pyrin domain containing 3 (NLRP3) is known to activate the inflammasome, a multi-protein platform leading to the processing of the IL-1 family of cytokines (11–14). NLRP3 inflammasome, which is composed of NLRP3, adaptor protein Apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1, is activated by mycobacteria (15–18). Following activation, inactive caspase-1 is processed by autocleavage via ASC and is converted into the active form of caspase-1 containing

10 kDa/20 kDa subunits. The active form of caspase-1 then cleaves pro-IL-1β and pro-IL-18 into mature forms of IL-1β and IL-18, respectively. The IL-1 family of cytokines, including IL-1β and IL-18, possess potent pro-inflammatory activities (19–21) and are responsible for the host defense against mycobacteria (22–29). However, several studies suggest that NLRP3 or caspase-1, which mediates the processing of the IL-1 family of cytokines, is not essential for the induction of protective immunity to *M. tuberculosis in vivo* (25, 26, 30–32). Thus, the signaling pathway leading to IL-1β/IL-18 production in mycobacterial infection remains controversial.

Recent studies identified that AIM2 (absent in melanoma 2), which possesses HIN-200 and pyrin domains, recognizes cytosolic DNA leading to activation of the inflammasome and secretion of IL-1β and IL-18 (33–36). Several studies have demonstrated that AIM2 is mandatory for the host defense against DNA viruses (Vaccinia virus and mouse cytomegalovirus) and intracellular bacteria (*Francisella tularensis* and *Listeria monocytogenes*) (37–41). However, the role of AIM2-dependent inflammasome activation in mycobacterial infection remains unknown.

In this study, we analyzed the role of AIM2 in mycobacterial infection. AIM2-deficient mice were highly sensitive to *M. tuberculosis* infection. AIM2-deficient macrophages showed impaired activation of the inflammasome and defective production of IL-1β and IL-18 after *M. tuberculosis* infection. Genomic DNA from *M. tuberculosis* was present within the cytosol after infection and induced activation of the inflammasome in an AIM2-dependent manner. These findings demonstrate the critical role of AIM2 in *M. tuberculosis* infection.

Methods

Mice and bacteria

The *Aim2* gene was isolated from genomic DNA extracted from embryonic stem cells (V6.5) by PCR using Elongase Enzyme Mix (Invitrogen). The targeting vector was constructed by replacing a 2.5-kb fragment encoding the exons of *Aim2* with a neomycin-resistance gene cassette and a herpes simplex virus thymidine kinase gene driven by the PGK promoter for negative selection. After transfection of the targeting vector into embryonic stem cells, colonies resistant to both G418 and ganciclovir were selected and screened by PCR and Southern blot. Homologous recombinants were microinjected into blastocysts of C57BL/6 female mice, and heterozygous F1 progenies were intercrossed to obtain AIM2-deficient mice. AIM2-deficient mice and their wild-type littermates from these intercrosses were used and all animal experiments were conducted with the approval of the Animal Research Committee of the Graduate School of Medicine at Osaka University.

Mycobacterium tuberculosis strain H37Rv (ATCC358121) was grown in Middlebrook 7H9-ADC medium for 2 weeks and stored at −80°C until use.

In vivo infection of mice

Mice were intratracheally infected with *M. tuberculosis* H37Rv (1×10^6 CFU per mouse). At 4 weeks after infection, homogenates of the lungs and livers were plated onto 7H10-OADC agar. For histological analysis, the lungs were fixed with 4% PFA, embedded in paraffin, cut into sections, and stained with hematoxylin and eosin or by the Ziehl–Neelsen method.

Harvest of BALF, blood and cells from tissues

Bronchoalveolar lavage fluid (BALF) was collected from uninfected and infected mice by washing the lung airways with phosphate buffered saline (PBS) at 3 weeks post-infection, and blood was collected from the hearts of uninfected and infected mice. $CD4^+$ T cells (4×10^5 cells) were isolated from the spleens at 3 weeks after infection, and then were stimulated with PPD (2 μg ml^{-1}; Japan BCG Laboratory) in the presence of APC (4×10^5 cells) for 48 h.

Mycobacterium tuberculosis genomic DNA extraction

Mycobacterium tuberculosis (1×10^9 CFU) was homogenized with glass beads (0.1 mm; ASONE) and proteins were removed using Phenol/Chloroform/Isoamyl alcohol and Chloroform/Isoamyl alcohol. Genomic DNA was precipitated using isopropanol.

Cell culture and stimulation

Mice were intraperitoneally injected with 4% thioglycollate (Sigma) and 3 days later macrophages were isolated from the peritoneal cavity. Cells were stimulated with LPS (200 ng ml^{-1}) for 3 h and then transfected with poly(dA:dT) (Sigma) or *M. tuberculosis* DNA using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Peritoneal macrophages were infected with *M. tuberculosis* (MOI of 3) for 6 h. Cells were washed three times with PBS and then incubated for 24 h.

ELISA

The concentration of IFN-γ, IL-1β or IL-12p40 in culture supernatants was measured by ELISA according to the manufacturer's instructions (R&D Systems). The ELISA kit for IL-18 was purchased from Medical & Biological Laboratories.

Immuno-precipitation and immuno-blot analysis

Supernatants were precleared with protein G–Sepharose (GE Healthcare), incubated with anti-caspase-1 p10 rabbit antibody (2 μg; Santa Cruz Biotechnology). Cell pellets were lysed in lysis buffer (1% Nonidet P-40, 150 mM NaCl and 50 mM Tris-HCl, pH 7.5) and together with the immuno-precipitants, separated on SDS-PAGE and transferred to PVDF membranes (Millipore). The membranes were incubated with anti-caspase-1 p10 antibody (1:200) and anti-β-actin antibody (1:500; Sigma). Bound antibody was detected with SuperSignal West Dura Extended Duration Substrate (Thermo).

Immuno-fluorescence analysis

Mycobacterium tuberculosis genomic DNA was labeled with Hoechst 33342 (100 ng ml^{-1}; Invitrogen) by incubation for 24 h. Hoechst-labeled *M. tuberculosis* was washed five times with PBS before use. RAW264.7 cells were infected with Hoechst-labeled *M. tuberculosis* (MOI of 3) for 6 h, washed three times with PBS and then incubated for 24 h. Cells were fixed with 4% PFA and permeabilized with 0.4% saponin. The cells were incubated with rabbit anti-Rab7 antibody (1:200; Santa Cruz Biotechnology) or rabbit anti-AIM2 antibody (1:200; Santa Cruz Biotechnology) or rat anti-LAMP1 antibody (1:200; BD Biosciences) for 1 h at room temperature. Cells

were then incubated with Alexa 488 anti-rabbit IgG antibody (Invitrogen) or Alexa 594 anti-rat IgG antibody (Invitrogen) for 40 min at room temperature. The immuno-stained cells were mounted with ProLong Gold antifade reagent (Invitrogen) on glass slides and analyzed using a fluorescence microscope (FV1000-D IX81; Olympus).

Statistical analysis

Differences between control and experimental groups were evaluated using Student's *t* test. Values of $P < 0.05$ were considered to indicate statistical significance.

Results

Aim2$^{-/-}$ mice are highly susceptible to Mycobacterium tuberculosis infection

To assess the *in vivo* role of AIM2, we generated AIM2-deficient mice by gene targeting (Supplementary Figure 1A is available at *International Immunology* Online), which was confirmed by Southern and northern blot analyses (Supplementary Figure 1B and C is available at *International Immunology* Online). *Aim2$^{-/-}$* mice were born at normal Mendelian ratios, developed normally and showed no apparent abnormalities when housed in our specific pathogen-free facility. Several previous studies demonstrated that AIM2 recognizes cytosolic DNA, leading to caspase-1 activation and subsequent processing of the IL-1 family of cytokines, such as IL-1β and IL-18 (33–36, 39). Therefore, we first analyzed the response to synthetic B-form double-stranded DNA [poly(dA:dT)] in *Aim2$^{-/-}$* macrophages. Peritoneal macrophages were collected from wild-type and *Aim2$^{-/-}$* mice and then transfected with poly(dA:dT) into the cytosol after priming with LPS. In immuno-blot analysis, a 10 kDa active form of caspase-1 (p10) was detected in wild-type macrophages stimulated with poly(dA:dT). In contrast, the cleaved p10 form of caspase-1 was not detected in *Aim2$^{-/-}$* macrophages (Supplementary Figure 2A is available at *International Immunology* Online). In addition, poly(dA:dT)-induced secretion of IL-1β and IL-18 into the culture supernatants was markedly decreased in *Aim2$^{-/-}$* macrophages, although ATP-induced secretion of IL-1β was normally observed (Supplementary Figure 2B is available at *International Immunology* Online). Thus, in accordance with previous reports, *Aim2$^{-/-}$* mice showed defective DNA-induced activation of the inflammasome.

We examined the involvement of AIM2 in mycobacterial infection using these *Aim2$^{-/-}$* mice. Wild-type and *Aim2$^{-/-}$* mice were intratracheally infected with the virulent H37Rv strain of *M. tuberculosis* and monitored for their survival (Fig. 1A). All *M. tuberculosis*-infected wild-type mice survived to at least 8 weeks post-infection. In contrast, all *Aim2$^{-/-}$* mice died within 7 weeks of infection with *M. tuberculosis*. We also assessed bacterial burdens in the lungs and livers at 4 weeks post-infection (Fig. 1B). Colony-forming unit titers of *M. tuberculosis* in lungs and livers were higher in *Aim2$^{-/-}$* mice than in wild-type mice. We next performed histological analysis of the lungs of mice at 4 weeks post-infection. Gross appearances of the lungs of wild-type and *Aim2$^{-/-}$* mice were markedly different, and many granulomatous changes were evident in *M. tuberculosis*-infected *Aim2$^{-/-}$* mice (Fig. 2A). Hematoxylin and eosin staining

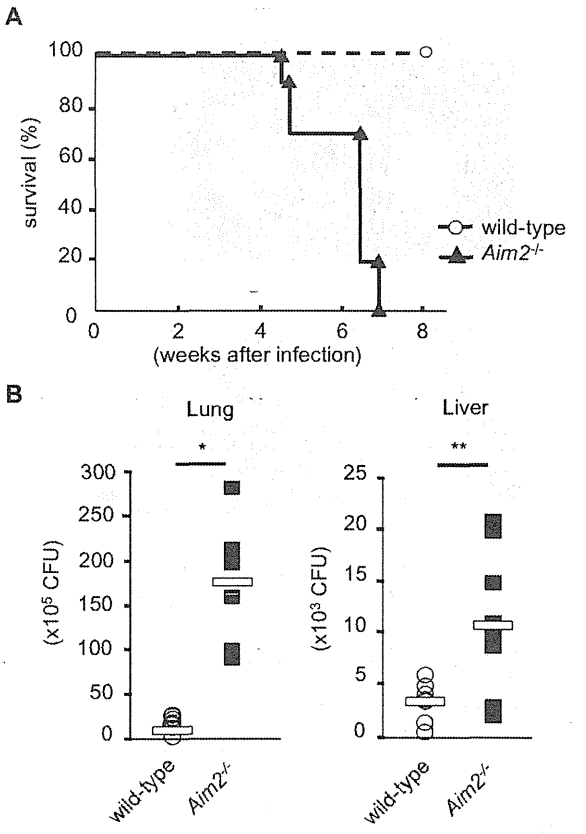

Fig. 1. *Aim2$^{-/-}$* mice are highly sensitive to infection with *M. tuberculosis*. (A) Wild-type ($n = 11$) and *Aim2$^{-/-}$* ($n = 10$) mice were intratracheally infected with *M. tuberculosis* and monitored for their survival. (B) Wild-type ($n = 7$) and *Aim2$^{-/-}$* ($n = 8$) mice were intratracheally infected with *M. tuberculosis*. At 4 weeks after infection, homogenates of the lungs and livers were plated onto 7H10-OADC agar and the CFU titers were counted. Symbols represent individual mice, and bars represent the mean CFU numbers. *, $P < 0.001$; **, $P < 0.01$.

of lung sections from infected *Aim2$^{-/-}$* mice demonstrated infiltration of many inflammatory cells (Fig. 2B). The number of *M. tuberculosis* in the lungs was measured by staining acid-fast bacilli using the Ziehl–Neelsen method (Fig. 2C). In the lungs of *Aim2$^{-/-}$* mice, the number of red-stained *M. tuberculosis* was markedly increased compared with those of wild-type mice. Taken together, these findings demonstrate that *Aim2$^{-/-}$* mice are highly susceptible to intratracheal infection with the virulent H37Rv strain of *M. tuberculosis*.

AIM2 mediates IL-1β/IL-18 production and T_h1 responses in Mycobacterium tuberculosis infection

Recent studies demonstrated that IL-1β is produced from *M. tuberculosis*-infected monocytes and alveolar macrophages mediating the host defense to mycobacteria (22–26, 42). Therefore, we first analyzed the levels of IL-1β in BALF from *M. tuberculosis*-infected mice (Fig. 3A). At 3 weeks after *M. tuberculosis* infection, IL-1β was abundantly detected in BALF from wild-type mice. In contrast, the concentration of IL-1β was profoundly decreased in BALF from *Aim2$^{-/-}$* mice. In addition to IL-1β, IL-18 has also been shown to be important for host resistance to mycobacterial infection (27–29). Therefore, we next

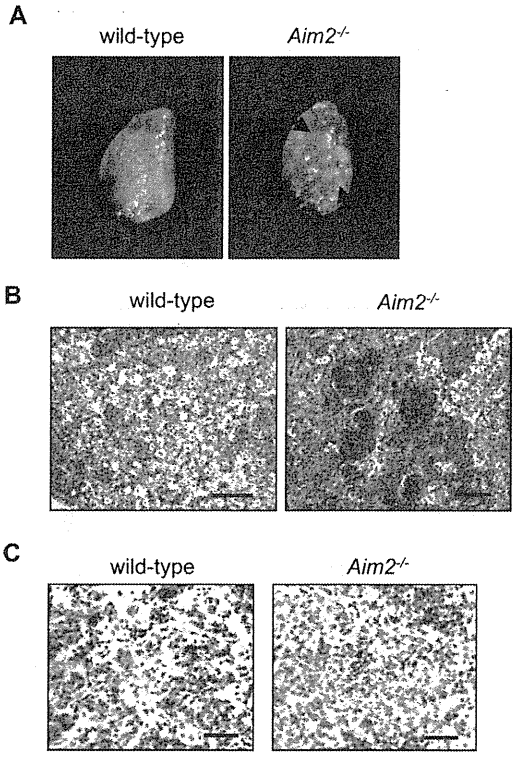

Fig. 2. High susceptibility of *Aim2*$^{-/-}$ mice to infection with *M. tuberculosis*. (A) Lung tissues from wild-type and *Aim2*$^{-/-}$ mice at 4 weeks post-intratracheal infection with *M. tuberculosis*. Arrows are shown to granulomatous lesion. (B) Lung tissue sections were stained with hematoxylin and eosin. Scale bars represent 100 μm. (C) Lung tissue sections were stained by the Ziehl–Neelsen method. Scale bars represent 40 μm.

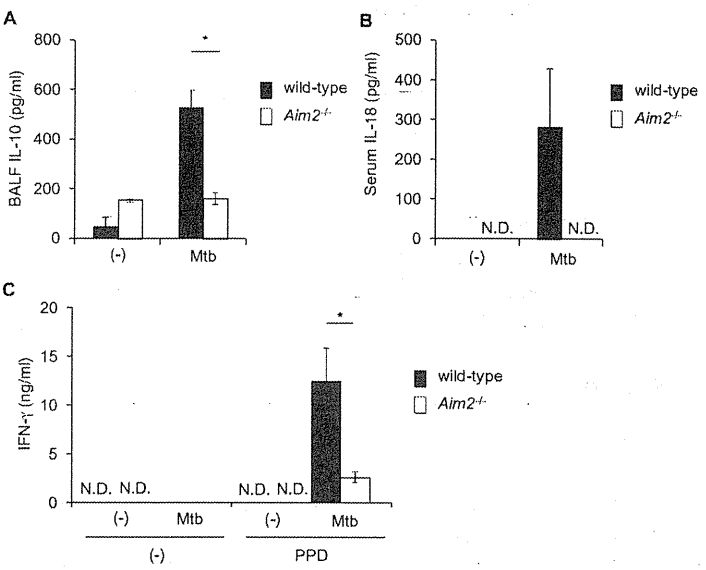

Fig. 3. Impaired production of IL-1β and IL-18 in *M. tuberculosis*-infected *Aim2*$^{-/-}$ mice. (A) BALF was collected from uninfected and infected mice at 3 weeks post-mycobacterial infection. BALF concentration of IL-1β was measured by ELISA. Data are presented as means ± SD of triplicate determinants and are one representative of two independent experiments. *, $P < 0.01$. (B) At 3 weeks after mycobacterial infection, sera were collected from uninfected and infected mice. Concentration of IL-18 was measured by ELISA. Data are presented as means ± SD of triplicate determinants and are one representative of two independent experiments. N.D., not detected. (C) CD4$^+$ T cells were isolated from the spleens of *M. tuberculosis*-uninfected and infected mice. The cells were co-cultured with APC for 48 h in the presence of PPD. The levels of IFN-γ in the cell supernatants were measured by ELISA. Data are presented as means ± SD of triplicate determinants and are one representative of two independent experiments. *, $P < 0.05$. N.D., not detected.

measured the serum levels of IL-18 in *M. tuberculosis*-infected mice (Fig. 3B). Serum concentration of IL-18 was increased in wild-type mice at 3 weeks post-infection. In contrast, IL-18 was not detected in sera from *M. tuberculosis*-infected *Aim2*$^{-/-}$ mice. We also assessed antigen-specific T_h1 responses following infection. CD4$^+$ T cells were isolated from the spleens of wild-type and *Aim2*$^{-/-}$ mice at 4 weeks post-infection and stimulated with a mycobacterial-specific antigen [purified protein derivative (PPD) of *Mycobacterium bovis*] in the presence of antigen-presenting cells (Fig. 3C). PPD stimulation induced marked production of IFN-γ in *M. tuberculosis*-infected wild-type mice. Antigen-specific production of IFN-γ was severely reduced in CD4$^+$ T cells derived from *M. tuberculosis*-infected *Aim2*$^{-/-}$ mice. These results indicate that the absence of AIM2 results in impaired production of IL-1β and IL-18 as well as T_h1 responses after *M. tuberculosis* infection.

AIM2 mediates Mycobacterium tuberculosis-induced inflammasome activation

We assessed the activation of caspase-1 to determine how AIM2-dependent immune responses develop following *M. tuberculosis* infection (Fig. 4A). Cleaved p10 form of caspase-1 was detected in *M. tuberculosis*-infected macrophages of wild-type mice. In contrast, cleavage of caspase-1 was severely reduced in *M. tuberculosis*-infected *Aim2*$^{-/-}$ macrophages.

We also analyzed cytokine production in *M. tuberculosis*-infected macrophages. Peritoneal macrophages from wild-type and *Aim2*$^{-/-}$ mice were infected with *M. tuberculosis* and the levels of cytokines in culture supernatants were measured by ELISA (Fig. 4B). *M. tuberculosis*-induced production of IL-12p40 or IFN-γ was comparable between wild-type and *Aim2*$^{-/-}$ macrophages. However, the production of IL-1β and IL-18 was severely reduced in *Aim2*$^{-/-}$ macrophages. Similar mRNA expression levels of *Il1b* and *Il18* in *M. tuberculosis*-infected wild-type and *Aim2*$^{-/-}$ macrophages were detected using real-time quantitative RT-PCR (Supplementary Figure 3 is available at *International Immunology* Online), indicating that AIM2 controls the production of IL-1β and IL-18 at the post-transcriptional level. Induction of *Ifnb*, encoding IFN-β, was enhanced in *M. tuberculosis*-infected *Aim2*$^{-/-}$ macrophages, confirming previous results (37). Thus, *M. tuberculosis*-infected *Aim2*$^{-/-}$ macrophages show defective caspase-1 activation leading to selective impairment in IL-1β and IL-18 secretion.

Mycobacterium tuberculosis genomic DNA activates AIM2 inflammasome

AIM2 has been shown to recognize cytosolic DNA (33–41). Therefore, we tested whether genomic DNA purified from

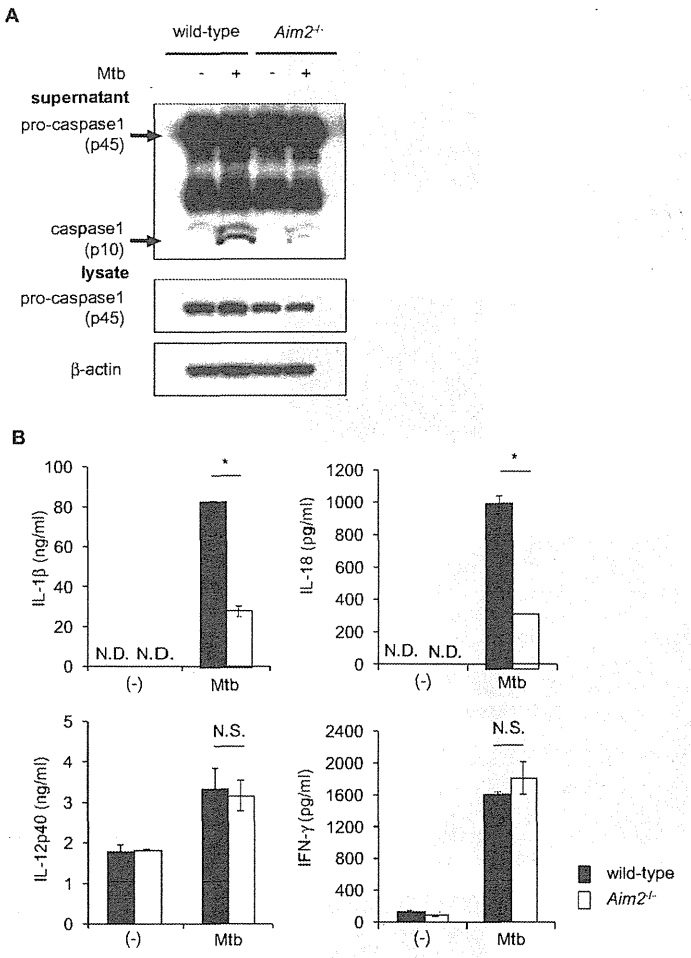

Fig. 4. AIM2-dependent inflammasome activation in *M. tuberculosis* infection. (A) *M. tuberculosis*-infected peritoneal macrophage culture supernatants were immuno-precipitated and the cell pellets were lysed. Caspase-1 specific bands were detected by western blotting and β-actin was used as a control for the cell lysate. One representative of three independent experiments is shown. (B) Thioglycollate-induced peritoneal macrophages were infected with *M. tuberculosis* (MOI of 3). The levels of the indicated cytokines in the culture supernatants were measured by ELISA. Data are presented as means ± SD of triplicate determinants and are one representative of three independent experiments. *, $P < 0.01$. N.S., not significant; N.D., not detected.

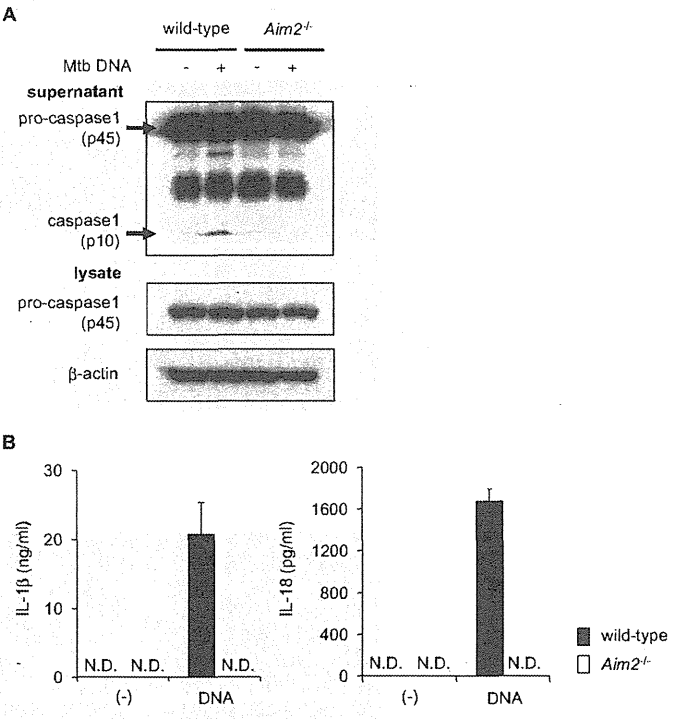

Fig. 5. AIM2 inflammasome is activated by *M. tuberculosis* genomic DNA. (A) Peritoneal macrophages were stimulated with LPS and transfected with Mtb DNA using Lipofectamine 2000. The culture supernatants were immuno-precipitated and the cells were lysed. Caspase-1 specific bands were detected by western blotting. β-actin was used as a control for the cell lysate. One representative of three independent experiments is shown. (B) Thioglycollate-elicited peritoneal macrophages were stimulated with LPS and transfected with Mtb DNA using Lipofectamine 2000. The production of IL-1β and IL-18 in the culture supernatants was measured by ELISA. Data are presented as means ± SD of triplicate determinants and are one representative of three independent experiments. N.D., not detected.

M. tuberculosis (Mtb DNA) activates caspase-1. Mtb DNA was transfected into the cytoplasm of peritoneal macrophages primed with LPS and analyzed for caspase-1 activation (Fig. 5A). The cleaved p10 form of caspase-1 was detected in LPS/Mtb DNA-stimulated wild-type macrophages. In contrast, the active form of caspase-1 was not induced in *Aim2*$^{-/-}$ macrophages stimulated with LPS/Mtb DNA. We also analyzed the secretion of IL-1β and IL-18 (Fig. 5B). Wild-type peritoneal macrophages stimulated with LPS/Mtb DNA secreted substantial amounts of IL-1β and IL-18. In contrast, the production of IL-1β and IL-18 was profoundly reduced in *Aim2*$^{-/-}$ macrophages. These findings indicate that AIM2 mediates Mtb DNA-dependent induction of caspase-1 activation and subsequent IL-1β/IL-18 secretion.

Mycobacterium tuberculosis genomic DNA is co-localized with cytosolic AIM2

We next determined the cellular compartment where Mtb DNA is recognized by AIM2. Recent studies demonstrated that virulent *M. tuberculosis* escapes from phagosomes into the cytosol (43). *Mycobacterium tuberculosis* was incubated with Hoechst 33342 to label genomic DNA and then infected into RAW264.7 macrophages. Some Mtb DNA was not co-localized with Rab7, which is recruited to the phagosomal membrane (Fig. 6A). Although LAMP1 is enriched in the phagolysosomal compartment, some Mtb DNA was not co-localized with it (Fig. 6B, upper panels). In contrast, genomic DNA from non-virulent *M. bovis* bacillus Calmette-Guerin (BCG) was fully merged with phagosome markers, Rab7 and LAMP1 (Supplementary Figure 4 is available at *International Immunology* Online). These findings indicate that Mtb DNA is present in the cytosol. We further visualized cytosolic AIM2 (Fig. 6B, lower panels). Mtb DNA, which was not present within the phagosome, co-localized with AIM2. We also analyzed co-localization of AIM2 and Mtb DNA in peritoneal macrophages (Supplementary Figure 5 is available at *International Immunology* Online). As was the case in RAW264.7 macrophages, Mtb DNA was merged with

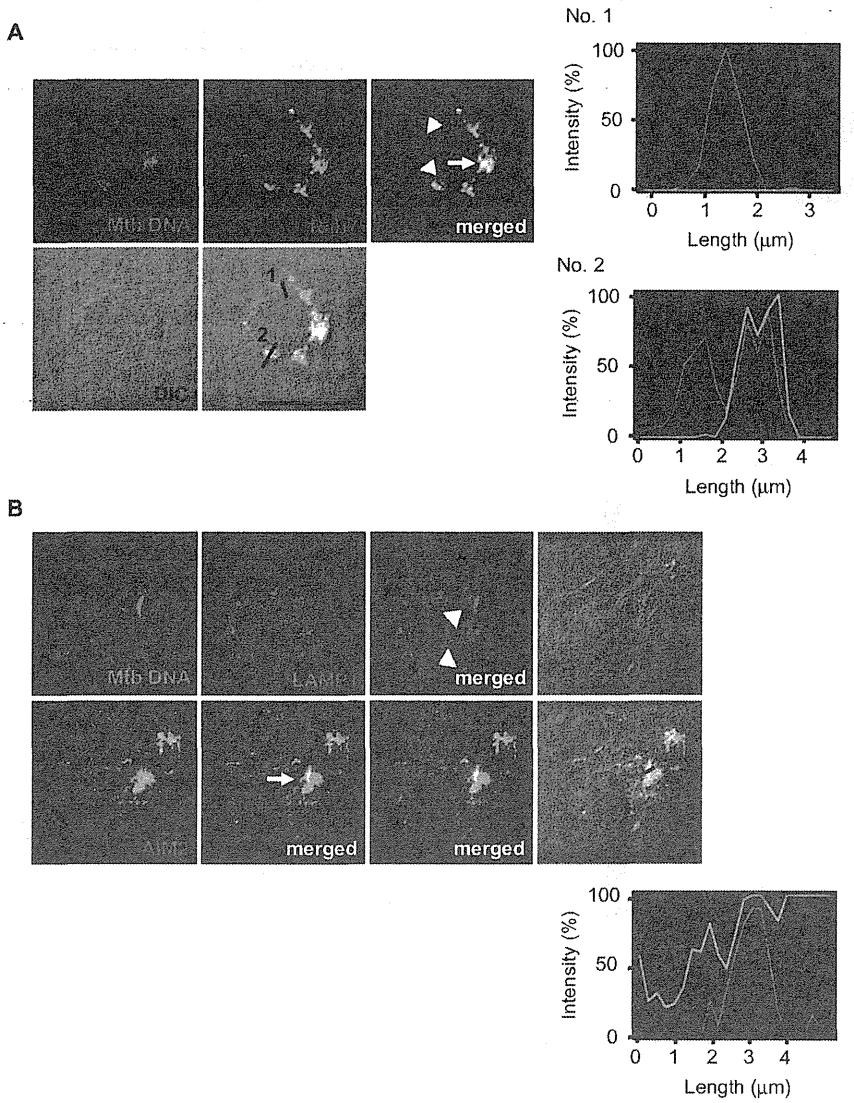

Fig. 6. Co-localization of cytosolic *M. tuberculosis* genomic DNA with AIM2. (A) RAW264.7 macrophages were infected with Hoechst-labeled *M. tuberculosis* (red) and stained with antibody to Rab7 (green). Scale bar represents 20 μm. Arrow heads indicate localization of the red signal alone. Arrow indicates co-localization of the red and green signals. Fluorescence intensities of the red and green signals were quantified along selected lines 1 or 2 (no. 1, upper panel; no. 2, lower panel). (B) RAW264.7 macrophages were infected with Hoechst-labeled *M. tuberculosis* (red) and stained with antibody to LAMP1 (blue, upper panels) or AIM2 (green, lower panels). Scale bar represents 20 μm. Arrow heads indicate localization of the red signal alone. Arrow indicates co-localization of the red and green signals. Fluorescence intensities of the blue, red and green signals were quantified along a selected line.

AIM2, but not with LAMP1. These findings demonstrate that the recognition of Mtb DNA by AIM2 occurs within the cytosolic compartment.

Discussion

In this study, we analyzed the role of AIM2 in the host defense against *M. tuberculosis* using AIM2-deficient mice. *Aim2*$^{-/-}$ mice were highly susceptible to mycobacterial infection compared with wild-type mice and showed severely reduced production of IL-1β and IL-18. IL-1β plays an important role in anti-mycobacterial host defense responses (22–26), and IL-18 is responsible for resistance to *M. tuberculosis* infection via induction of IFN-γ-mediated T_h1 responses (27–29). Therefore, defective production of IL-1 family cytokines, such as IL-1β and IL-18, might cause a high sensitivity to *M. tuberculosis* infection in *Aim2*$^{-/-}$ mice.

Mycobacterium tuberculosis has been shown to activate the inflammasome via NLRP3 (15–18). However, several studies showed that mice deficient in NLRP3 are not highly sensitive to *M. tuberculosis* infection (26, 30–32). This study clearly shows that *Aim2*$^{-/-}$ mice are highly sensitive to infection with *M. tuberculosis* with defective activation of caspase-1, identifying AIM2 as the important molecule for activation of the inflammasome in *M. tuberculosis* infection. However, mice deficient in ASC or caspase-1,

critical components of the inflammasome, do not show dramatically severe phenotypes as compared with those of IL-1β-deficient mice in *M. tuberculosis* infection (25, 26). In this regard, AIM2 might also activate a signaling pathway, leading to inflammasome-independent production of the IL-1 family of cytokines. *Mycobacterium tuberculosis*-infected *Aim2*$^{-/-}$ macrophages expressed high amounts of IFN-β, confirming a previous study that showed that poly(dA:dT) induces increased amounts of IFN-β in *Aim2*$^{-/-}$ splenocytes and macrophages (37). Thus, AIM2 might be involved in the signaling pathway responsible for suppression of IFN-β. IFN-β is induced by *M. tuberculosis* infection and then suppresses the production of IL-1β in macrophages and dendritic cells (44,45). Indeed, the type I IFNs have been shown to contribute to impaired host resistance to *M. tuberculosis* in mice (26, 46–48). Thus, it is possible that AIM2 activates two signaling pathways, one mediating the inflammasome-dependent processing of the IL-1 family of cytokines and a second that mediates activation of unknown pathways that sustain the production of IL-1β by suppressing type I IFNs.

The cleaved p10 form of caspase-1 was not detected, and production of IL-1β and IL-18 was almost completely abrogated in Mtb DNA-stimulated *Aim2*$^{-/-}$ macrophages. In contrast, IL-1β and IL-18 were moderately produced and the cleaved form of caspase-1 was also slightly detected in *M. tuberculosis*-infected *Aim2*$^{-/-}$ peritoneal macrophages. This might be due to NLRP3-dependent recognition of *M. tuberculosis* (15–18). An AIM2-independent and inflammasome-independent mechanism for IL-1β release might also be operating in mycobacterial infection. Matrix metalloproteinase-9 (MMP-9, also known as gelatinase B) is robustly induced in mycobacteria-infected macrophages, causing inactive IL-1β to be processed into a biologically active form (49, 50). Thus, MMP-9 might be involved in the inflammasome-independent processing of the IL-1 family of cytokines during *M. tuberculosis* infection.

We showed that Mtb DNA co-localized with AIM2 in the cytosol, but the localization of mycobacteria within macrophages is still under debate (51). Virulent *M. tuberculosis* resides within phagosomes by inhibiting their maturation into phagolysosomes (52–54). But there are several reports supporting cytosolic escape of virulent mycobacteria (43, 51, 55–57). *Mycobacterium marinum* can escape from phagosomes into the cytosol by actin-based motility (55), an activity which depends on the region of difference 1 [RD1 (57)]. *Mycobacterium tuberculosis* and *Mycobacterium leprae* can translocate from phagosomes to the cytosol of myeloid cells in an RD1-dependent manner and following cell death (43). This might be compatible with our results for *M. tuberculosis*-induced activation of the AIM2 inflammasome, which also requires induction of pyroptosis, a form of cell death (33–39). Our data suggest that *M. bovis* BCG, lacking the RD1 locus, fails to escape from phagosomes into the cytosol. In addition, *M. bovis* BCG does not induce IL-1β secretion from macrophages (32). Thus, AIM2 recognizes *M. tuberculosis* upon translocation into the cytosol. It will be interesting to determine how Mtb DNA becomes exposed and is recognized by AIM2 in the cytosol.

We have identified a novel recognition mechanism in mycobacterial infection through the cytosolic DNA sensor AIM2. Several pattern recognition receptors, such as TLRs and C-type lectin receptors mediate gene expression in mycobacterial infection. AIM2 is co-operatively involved in the immune response to mycobacterial infection with these innate immune mycobacterial sensors.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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ORIGINAL ARTICLE

Decreased plasma granulysin and increased interferon-gamma concentrations in patients with newly diagnosed and relapsed tuberculosis

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ABSTRACT

Granulysin and interferon-gamma (IFN-γ) have broad antimicrobial activity which controls *Mycobacterium tuberculosis* (*M. tuberculosis*) infection. Circulating granulysin and IFN-γ concentrations were measured and correlated with clinical disease in Thai patients with newly diagnosed, relapsed and chronic tuberculosis (TB). Compared to controls, patients with newly diagnosed, relapsed and chronic TB had lower circulating granulysin concentrations, these differences being significant only in newly diagnosed and relapsed TB ($P < 0.001$ and 0.004, respectively). Granulysin concentrations in patients with newly diagnosed and relapsed TB were significantly lower than in those with chronic TB ($P = 0.003$ and $P = 0.022$, respectively). In contrast, significantly higher circulating IFN-γ concentrations were found in patients with newly diagnosed and relapsed TB compared to controls ($P < 0.001$). The IFN-γ concentrations in newly diagnosed and relapsed patients were not significantly different from those of patients with chronic TB. However, *in vitro* stimulation of peripheral blood mononuclear cells (PBMCs) from patients with newly diagnosed, relapsed and chronic TB with purified protein derivative (PPD) or heat killed *M. tuberculosis* (H37Ra) enhanced production of granulysin by PBMCs. *In vitro*, stimulation of PBMCs of newly diagnosed TB patients with PPD produced greater amounts of IFN-γ than did controls, while those stimulated with H37Ra did not. The results demonstrate that patients with active pulmonary TB have low circulating granulysin but high IFN-γ concentrations, suggesting possible roles in host defense against *M. tuberculosis* for these agents.

Key words clinical disease, granulysin, IFN-γ, tuberculosis.

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List of Abbreviations: APC, antigen presenting cell; BCG, Bacillus Calmette-Guérin; CTL, cytotoxic T lymphocyte; E, ethambutol; H, isoniazid; IFN-γ, interferon gamma; IGRA, interferon-γ release assay; IL, interleukin; MDR, multi-drugs resistance; MHC, major histocompatibility complex; *Mtb*, *Mycobacterium tuberculosis*, *M. tuberculosis*, *Mycobacterium tuberculosis*; NK, natural killer; PBMC, peripheral blood mononuclear cell; PPD, purified protein derivative; R, rifampicin; S, streptomycin; TB, tuberculosis; Th1, T-helper type 1; TMB, tetramethylbenzidine; TNF, tumor necrosis factor; TST, tuberculin skin test; XDR, extensively drug resistant; Z, pyrazinamide.

Tuberculosis is a major health problem worldwide, with one third of the world population being infected and approximately 1.1–1.7 million deaths annually (1). Most individuals infected with *Mtb* are asymptomatic. However, 5–10% will progress to active TB during their lifetime, the remainder being resistant to active TB, but remaining infected. Relapse of TB, which is defined as an episode of infection occurring after a previous episode has been treated and considered cured, is possibly due to endogenous reactivation when it occurs in geographical areas with a low incidence of TB infection (2). However, generally the risk of relapse depends on the intensity of exposure to *Mtb*. Other factors that directly affect the clinical course of TB are host factors, including age, immune status, genetic factors and coinfection with HIV, and bacterial factors, including degree of exposure, virulence of strain, MDR and XDR.

Protective immunity against *Mtb* infection involves activated macrophages, antigen-specific T cells and type-1 cytokines such as IL-12, IFN-γ and TNF (3, 4). Inherited defects of the IL-12/IFN-γ pathway appear to result in a variety of changes in mycobacterial susceptibility. People with genetic deficiencies in the type-1 cytokine (IL-12/IL-23/IFN-γ) axis, and those with neutralizing autoantibody against IFN-γ, have been found to be highly susceptible to mycobacterial infections including TB (5–8). In active pulmonary TB, these effectors of the immune response are activated, as evidenced by observation of high circulating IFN-γ concentrations that decrease significantly following two months of therapy (9, 10).

Granulysin can kill extracellular *Mtb* directly, or intracellular bacteria in the presence of perforin (11), expression of granulysin in CD8+T cells being induced upon activation. It has recently been reported that granulysin is strongly associated with diverse activities of NK cells and CTLs in physiological and pathological settings, and might be a useful novel serum marker for evaluating the overall status of host cellular immunity (12). In patients with cutaneous leprosy, the frequency of granulysin-expressing T cells lesions is 6-fold greater than in those with the disseminated lepromatous form of the disease (13). In contrast, adults with active pulmonary TB in a highly TB endemic area in Indonesia had significantly lower plasma granulysin concentrations than did controls, these concentrations increasing after 2 months of anti-TB therapy to values similar to those of controls, and having increased even further after completion of anti-TB therapy. These changes in granulysin concentrations occurred predominantly in patients in whom IFN-γ negative T cells were expressed, suggesting that in TB the cellular sources of IFN-γ and granulysin are partly non-overlapping (14). Similar findings have been reported for Italian children, the lowest concentrations having been found in TB patients who were PPD negative at the time of diagnosis (15), indicating the involvement of granulysin and IFN-γ in curative immune responses against *Mtb*. In chronic pulmonary TB, lung tissue biopsy has shown reduction in amounts of perforin and granulysin in relation to granzyme A, while higher per cell expression of perforin and granulysin is associated with bacteriological control, suggesting that perforin and granulysin could be used as markers or correlates of immune protection in human TB (16). However, effective host mechanisms against *Mtb* infection are not well understood, this lack of understanding being a problem in regard to vaccine development and immunotherapy for TB. Moreover, so far there is limited information regarding the roles of IFN-γ and granulysin in recurrent TB. Therefore, the present study aimed to investigate whether granulysin and IFN-γ responses are associated with clinical disease in patients with newly diagnosed, relapsed and chronic pulmonary TB in northern Thailand, where TB is endemic.

MATERIALS AND METHODS

Subjects

One hundred and fifty-five pulmonary TB patients (aged 9 to 88 years) were recruited from the outpatient and inpatient clinics of Chiang Rai Hospital and Mae Chan Hospital, in the north of Thailand. These included 102 male and 53 female patients with newly diagnosed and previously treated pulmonary TB. Patients with extrapulmonary TB and pulmonary TB/HIV seropositive were excluded. All patients with pulmonary TB had clinical symptoms and a confirmed diagnosis on the basis of presence of acid-fast bacilli in sputum on microscopic examination, positive cultures of *Mtb*, medical history and chest radiographic findings. Patients were categorized according to World Health Organization criteria (1), which include ascertaining whether the patient has previously received TB treatment. The TB drug regimens were based on the recommendations of the National Tuberculosis Program, Ministry of Public Health, Thailand. Standard TB treatment drugs consist of streptomycin (S), isoniazid (H), rifampicin (R), pyrazinamide (Z) and ethambutol (E). In this study, patients with newly diagnosed TB were defined as those who had never received treatment for TB or had taken anti-TB drugs for less than 1 month prior to enrollment ($n = 84$). Patients with relapsed TB were defined as those previously treated for TB and declared "cured" or "treatment completed", and currently diagnosed as *Mtb* positive by smears and cultures ($n = 35$). Patients with chronic TB were defined as those who had started on a retreatment regimen after having failed previous treatment ($n = 36$). No patients had been reported to be MDR or

XDR cases on the basis of drug sensitivity tests at the time of enrollment in this study.

Thirty three healthy individuals (aged 21 to 54 years old, median = 36 years) recruited from the Blood Bank of Chiang Rai Hospital, Mae Chan Hospital and Phan Hospital were used as controls. They had no history suggestive of TB or other acute infectious diseases or diabetes at the time of enrollment. However, they were not subject to chest X-rays, TSTs or testing for latent TB infection and infection manifesting as active TB by IGRA upon enrollment.

The ethical aspects of this study were approved by the Ethical Review Committee for Research in Human Subjects, Ministry of Public Health, Thailand (Ref. No.3/2550) as part of a project studying multiple factors in recurrent TB, and written informed consent was obtained from all subjects.

Blood samples

Before instituting anti-TB therapy, blood was collected aseptically in EDTA Vacutainers. Plasma and packed cells were separated by centrifugation and stored at −80°C.

HIV screening

HIV positive cases were excluded from the study by screening with the particle agglutination assay (Serodia-HIV-1/2, Fujirebio, Tokyo, Japan) and/or immunochromatographic rapid test (Determine HIV-1/2, Abbott Laboratories, Champaign, IL, USA) or by ELISA (Enzygnost Anti-HIV 1/2 plus ELISA, Dade Behring, Marburg, Germany).

Peripheral blood mononuclear cells isolation and stimulation

Peripheral blood mononuclear cells from 75 pulmonary TB patients and 4 healthy controls were isolated by Ficoll-Hypaque density gradient centrifugation. In brief, 3 mL of whole blood in K_3EDTA (Greiner Bio-One, Bangkok, Thailand) was diluted with an equal volume of PBS, mixed gently and layered carefully over 3 mL Ficoll-paque PLUS (Amersham Biosciences, Uppsala, Sweden). After centrifugation at 1000 g for 20 min at room temperature, the PBMCs were harvested. The supernatant was removed after centrifugation at 700 g for 10 min at 4°C and the pellet adjusted with RPMI 1640 containing 10% FBS. The viable PBMCs were counted in 0.2% Trypan blue. Approximately 1×10^6 PBMCs/mL in RPMI 1640 medium containing 10% FBS and 2-mercapto ethanol were added to each well of a 24 well plate, stimulated either with 20 μg/mL of PPD (Japan BCG laboratory, Kiyose, Japan) or heat killed *Mtb* (H37Ra) (Difco, Detroit, MI, USA) and incubated at 37°C in 5% CO_2. The supernatants were harvested after 40 hr of stimulation, centrifuged at 1200 g for 3 min at 4°C and kept at −80°C. PMBCs stimulated with 20 μg/mL of PPD and not stimulated were used as positive and negative controls, respectively.

Determination of circulating granulysin and granulysin production by peripheral blood mononuclear cell stimulation assay

The granulysin concentrations in plasma and stimulated PBMC supernatant were determined by ELISA according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, CA, USA). The tests were done in duplicate. Briefly, a microtiter plate (Costar, Cambridge, MA, USA) was coated with 100 μL/well of 5 μg/mL monoclonal mouse anti-human granulysin (clone RB1) (MBL International, Nagoya, Japan) in 0.05 M carbonate-bicarbonate buffer (pH 9.5) overnight at 4°C. The plates were washed with PBS containing 0.05% Tween 20 and blocked with buffered protein solution with ProClin-150 at room temperature for 1 hr. After being washed, the undiluted plasma was added and incubated for 2 hr at room temperature. The bound antigens were detected with 0.1 μg/mL of monoclonal mouse anti-human granulysin biotin (RC8) (MBL International) and avidin-horseradish peroxidase (Av-HRP) conjugate (BD Biosciences Pharmingen) diluted to 1:1000. After incubation for 1 hr, the reactions were developed by coloring with TMB substrate (BD Biosciences Pharmingen) for 20 min in the dark. The reaction was stopped by 2N H_2SO_4 solution (BD Biosciences Pharmingen). Optical densities were measured at 450 nm wavelength by an ELISA reader (ELx808 IU ultra microplate reader, Bio-Tek instruments, Winooski, VT, USA). Granulysin concentrations were calculated from a standard curve using granulysin containing culture supernatant obtaining from Cos7 cell transfected with gene encoding 15K granulysin. The lower detection limit for granulysin was 0.047 ng/mL.

Determination of circulating interferon-γ concentrations and interferon-γ production from stimulated mononuclear cells *in vitro*

Interferon-γ concentrations in plasma and stimulated PBMC supernatant were determined by ELISA according to the manufacturer's instruction (BD Biosciences Pharmingen). The tests were done in duplicate. Briefly, a microplate (Costar) was coated with 100 μL/well of anti-human IFN-γ (diluted to 1:250 in 0.1 M sodium carbonate) and incubated overnight at 4°C. The plates were washed three times with PBS containing 0.05% Tween 20, blocked with 200 μL/well of buffered protein solution with ProClin-150 and incubated at room temperature for 1 hr. After being washed, 100 μL of undiluted sample was added and incubated for 2 hr at room temperature. The bound

antigen were detected with biotinylated anti-human IFN-γ monoclonal antibody and streptavidin-horseradish peroxidase conjugate (diluted to 1:250 with 10% FBS in PBS) and incubated for 1 hr at room temperature. Then, 100 μL of TMB substrate solution was added and incubated for 30 min at room temperature in the dark. The reaction was stopped by 2N H_2SO_4 solution. Samples were analyzed at 450/550 nm wavelength with a microplate ELISA reader (ELx808 IU ultra microplate reader) and IFN-γ concentrations were calculated from a standard curve using recombinant human IFN-γ. The lower detection limit was 4.7 pg/mL.

Statistical analyses

Statistical analyses were performed by SPSS software version 17.0. IFN-γ and granulysin concentrations in different independent subject groups were compared by Mann-Whitney U test. A P value < 0.05 was considered statistically significant.

RESULTS

Clinical characteristics of subjects

The clinical characteristics of the patients in the study with newly diagnosed, relapsed and chronic TB are summarized in Table 1. Infiltrates without cavitation were found on the chest radiographs of the majority of patients with newly diagnosed (57.1%) and relapsed TB (51.4%). Most patients with newly diagnosed TB (63.1%) were treated with category 1 drug regimens (2HRZE(S)/4HR) whereas relapsed (60%) and chronic TB patients (52.8%) were treated with category 2 drug regimens (2HRZES/1HRZE/5HRE). Treatment success ("cure" or "treatment completed") was achieved in 66.7%, 57.1% and 47.2% of patients with newly diagnosed, relapsed and chronic TB, respectively. Nine chronic TB patients (25.0%) had microscopically positive sputum smears at the end of their treatment course, indicating treatment failure. The median treatment duration was 7 months in patients with newly diagnosed and relapsed TB and 9 months in those with chronic TB.

Table 1. Characteristics and clinical profile of study subjects

Characteristic	Newly diagnosed TB $N = 84$	Relapsed TB $N = 35$	Chronic TB $N = 36$
Sex			
Male	60	27	15
Female	24	8	21
Age (years)			
Median	44	48	49
Range	9–85	28–88	14–82
Chest X-ray findings			
Infiltrate/Non-cavitating	48	18	15
Cavitating	14	8	6
Not documented	22	9	15
Treatment regimens			
2HRZE(S)/4HR[a] (CAT1)	53	10	
2HRZES/1HRZE/5HRE[a] (CAT2)	19	21	19
2HRZ/2HR[a] (CAT3)			
Second line drug (CAT4)	12	4	17
Duration of treatment (months)			
Median	7	7	9
Range	0–26	0–14	5–20
Treatment outcomes			
Cure	51	18	14
Completed	5	2	3
Default	10	5	7
Died	4	6	3
Failure	7	3	9
Not documented	7	1	

[a]The standard code for TB treatment regimens, each anti-TB drug has an abbreviation: streptomycin (S), isoniazid (H), rifampicin (R), pyrazinamide (Z) and ethambutol (E). CAT, category.

Circulating granulysin concentrations in clinical tuberculosis before anti-tuberculosis therapy

The concentrations of circulating granulysin in patients with newly diagnosed TB (median ± SE = 1.511 ± 0.287 ng/mL, range 0.560–15.600 ng/mL) and relapsed TB (median ± SE = 1.458 ± 0.329 ng/mL, range 0.403–8.110 ng/mL) were significantly lower than those of healthy controls (median ± SE = 2.470 ± 0.186 ng/mL, range 0.662–5.055 ng/mL) ($P < 0.001$, $r = -3.816$ and $P = 0.004$, $r = -2.853$, respectively). Patients with chronic TB (median ± SE = 1.917 ± 0.264 ng/mL, range 0.549–6.970 ng/mL) had lower granulysin concentrations than controls, this difference not being significant ($P = 0.442$, $r = -0.769$). Median concentrations of granulysin were similar in patients with newly diagnosed and relapsed TB, but both were significantly lower than in chronic TB ($P = 0.003$, $r = -2.967$ and $P = 0.022$, $r = -2.294$, respectively) (Fig. 1).

Granulysin production in peripheral blood mononuclear cell stimulation assay

Granulysin production in PBMCs stimulated *in vitro* with PPD and H37Ra were measured in 46 patients with newly diagnosed, 21 with relapsed and 8 with chronic TB. Granulysin production by newly diagnosed TB-PBMCs stimulated *in vitro* with PPD (median ± SE = 0.796 ± 0.071 ng/mL, range 0.208–2.196 ng/mL) and H37Ra (median ± SE = 0.976 ± 0.065 ng/mL, range 0.246–1.823 ng/ml) were significantly higher than those of