THE JOURNAL OF DERMATOLOGY

doi: 10.1111/1346-8138.12047 *The Journal of Dermatology* 2012; **40**: 1–9

REVIEW ARTICLE

Laboratory procedures for the detection and identification of cutaneous non-tuberculous mycobacterial infections

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ABSTRACT

There is evidence that the incidence of cutaneous non-tuberculous mycobacterial (NTM) infection is increasing worldwide. Novel culture methods and new analytical procedures have led to significant advancements in understanding the origin and progression of NTM infections. Differential identification of NTM isolates is important because culture characteristics and/or sensitivity to anti-mycobacterium drugs vary between different mycobacterial species. In this manuscript, we describe the latest diagnostic techniques for cutaneous NTM infection and show how these methodologies can be used for the diagnosis of Buruli ulcer in Japan.

Key words: 16S rRNA gene, Buruli ulcer, cutaneous infection, non-tuberculous mycobacterial infection, polymerase chain reaction, species identification.

INTRODUCTION

The basis for the increase in the number of cases of cutaneous non-tuberculous mycobacterial (NTM) infection is unknown. It has been attributed to an increase in the total number of these patients or to better detection and/or reporting methods.[1–5] Insight into the origin and progression of NTM infections has been significantly advanced due to the employment of novel methods for the culture and analysis of NTM. However, the diagnosis of a cutaneous NTM infection in less experienced hospitals is occasionally delayed, or not made at all, because the causative agent can be difficult to isolate due to its variety of growth characteristics. To date, most cutaneous NTM infections have been caused by *Mycobacterium marinum*[6,7] or by groups of rapidly growing mycobacterial (RGM) species, but sometimes a rare mycobacterium can cause the disease.[8–10] Different strains of mycobacteria generally exhibit different characteristics in culture and/or sensitivity to anti-mycobacterial drugs. Therefore, accurate identification of the causative agent is important for the treatment of NTM infections. In this manuscript, we describe the latest molecular diagnostic techniques for cutaneous NTM infection and present a case study that used these methodologies for BU diagnosis.

NTM IN DERMATOLOGY

Causative agents of dermatological infections

Approximately 30 mycobacterial species have been identified as causative agents of cutaneous infection (Table 1). These species of mycobacteria, with the exception of the *Mycobacterium tuberculosis* complex and *Mycobacterium leprae*, are classified as NTM. They are categorized into four groups based on their growth rate (slow growing mycobacterial [SGM] and RGM species) and photochromogenicity. The identification of mycobacterial species is now rapid and relatively simple. Some of the pathogens identified as causative agents are used as a designation of the disease (e.g. cutaneous *Mycobacterium massiliense* infection).

Clinical symptoms of cutaneous NTM infection

A number of cutaneous diseases, such as erythema, nodules, erosion and ulcers, have been attributed to NTM infection. The point of entry is thought to be via minute cutaneous wounds in which bacteria are attached. Although a visceral NTM infection may cause cutaneous infection in immunocompromised patients, it is not yet clear how many bacteria are required for pathogenicity. There is usually only one cutaneous lesion; however, multiple skin lesions have been observed when pathogenic microbes spread through the lymph fluid. Most infected skin lesions are on exposed areas such as the hands, feet or face, supporting the premise that small external wounds are the penetration pathway. For example, in cutaneous *M. marinum* infection, also known as fish tank granuloma, approximately 80% of the patients have eruption(s) in finger, hand and/or wrist joints. Patients do not experience the pain or itching usually associated with eruptions, but inflammation does sometimes lead to tenderness and/or spontaneous pain.[11,12]

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Received 7 September 2011; accepted 24 October 2012.

Table 1. Causative agents of cutaneous mycobacterial infections

Growth rate	Traditional Runyon classification	Species (*Mycobacterium*)	Disease
Slow growers	*M. tuberculosis* complex	*M. tuberculosis* *M. bovis*	Cutaneous tuberculosis
	Group I Photochromogens	*M. kansasii* *M. marinum* *M. simiae*	Non-tuberculous mycobacterial infection
	Group II Scotochromogens	*M. gordonae* *M. scrofulaceum* *M. szulgai* *M. ulcerans* subsp. *shinshuense*[a] *M. ulcerans*[a]	
	Group III Non-photochromogens	*M. avium* *M. haemophilum* *M. intracellulare* *M. xenopi*[b]	
Rapid growers	Group IV Rapid growers	*M. abscessus* *M. chelonae* *M. fortuitum* *M. peregrinum* *M. vaccae*	
Unculturable in artificial medium		*M. leprae*	Hansen's disease (Leprosy)

[a]Yellow pigmentation is sometimes lost after several passages. [b]Non-pigmented colonies during early growth. However, most colonies become yellow with age.

CONVENTIONAL IDENTIFICATION OF CUTANEOUS NTM

Specimens

Pus or skin exudate, skin scrapings and skin biopsies are the major source of samples used to perform both conventional and molecular mycobacterial assays (Table 2).[13,14] Isolates, formalin-fixed and/or formalin-fixed paraffin-embedded sections are also used for analysis. Animal coats, aquatic animals, seawater, tap water and swimming pool water are also used in the search for sources of infection. To date, we have received 90 clinical specimens (swabs and tissues) for NTM detection and identification, 225 cultured colonies for identification, 31 paraffin-embedded specimens for detection and 278 NTM cultured colonies for drug susceptibility assay from April 2009 to March 2011. As we discuss later, we have been using this pipeline since 2006, and these samples were analyzed at least in part to reach the correct identification.

Smear test and pathological test

Swabs containing pus or skin exudate spread on a slide glass is used for the smear tests. Smears are stained (Ziehl–Neelsen [Z-N] or Auramine O staining) and examined by light microscopy or fluorescent microscopy (Fig. 1). Pathology specimens are also stained with Z-N staining (Fig 2).

Culture test

Liquid or solid medium is used to culture samples obtained from swabs containing pus or exudate, skin brushes and skin biopsies. The major liquid medium has a Middlebrook 7H9 Broth base combined with a growth indicator system (Mycobacteria Growth Indicator Tube, MB/BacT).[15,16] Three representative solid media are the egg-based Löwenstein–Jensen,

Table 2. Laboratory procedures to detect and identify cutaneous NTM

- Pus or scrapings
  - PCR
  - Smear test (Z-N stain)
  - Culture at room temperature: L-J or Ogawa medium
  - Culture at 37°C: liquid medium (e.g. MGIT)
  - Frozen in −20°C
- Biopsy samples
  - PCR
  - Pathological test (Z-N stain)
  - Culture at room temperature: L-J or Ogawa medium
  - Culture at 37°C: liquid medium (e.g. MGIT)
  - Frozen in −20°C
    - PCR
    - Culture
- Cultured samples
  - PCR
  - DDH
  - Biochemical assays
  - Drug susceptibility assays
- Paraffin- embedded materials
  - PCR

DDH, DNA–DNA hybridization; L-J medium, Löwenstein–Jensen medium; MGIT, Mycobacteria Growth Indicator Tube;[15] NTM, non-tuberculous mycobacterial; PCR, polymerase chain reaction; Z-N stain, Ziehl–Neelsen stain.

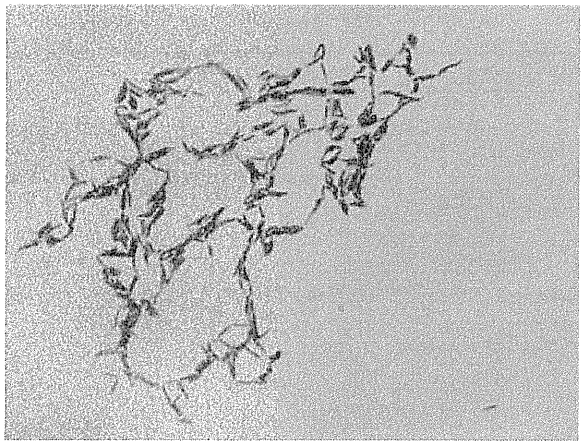

Figure 1. Smear Ziehl–Neelsen staining (original magnification ×1000).

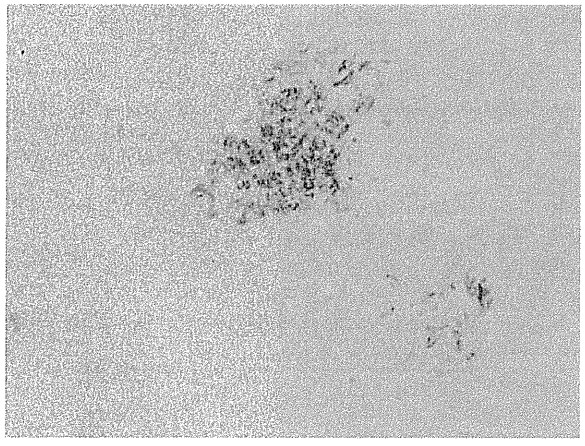

Figure 2. Pathological Ziehl–Neelsen staining (original magnification ×400).

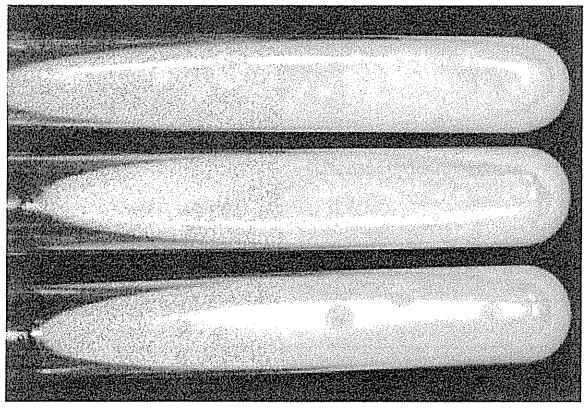

Figure 3. Ogawa medium (culture positive).

Ogawa medium (Fig. 3), or Middlebrook 7H10/7H11 agar medium.[17] Before culturing, pretreatment with Nalc-NaOH is necessary to avoid contamination by other bacteria or fungi. Optimal culturing temperature varies depending on species: 40°C, 37°C, 33°C, 30°C, 28°C and room temperature are frequently used. If multiple incubators are not available for simultaneous use, incubation at 37°C is recommended with simultaneous culturing at room temperature. The isolation period of the causative agents may be shortened when liquid media is used. However, the shortened growth period may not allow sufficient time to observe colony characteristics. Because liquid medium is not suitable for identification when samples contain multiple pathogens, it is recommended that both liquid and solid cultures be grown at the same time.

Biochemical analysis

Biochemical analyses such as the niacin and catalase tests and other enzymatic reaction assays have been the most frequently performed procedures for mycobacterial species identification. These biochemical tests have certain limitations that lead some laboratories to avoid them. For example, testing can only be performed after successful isolation. In addition, running the tests requires complicated quality control and technical expertise.[13,14]

MOLECULAR IDENTIFICATION OF CUTANEOUS NTM

Differential diagnosis by DNA–DNA hybridization (DDH) assays

Differential diagnosis using a commercial kit for mycobacterial DDH can be performed when bacterial isolates are available.[18] The kit contains a panel of 18 major mycobacteria (Table 3). However, it is impossible to diagnose a rare species and subspecies that is not included in the panel, and there have

Table 3. Mycobacterial species identifiable in a commercially available DNA–DNA hybridization test

TB complex (*M. africanum*, *M. bovis*, *M. microti*, *M. tuberculosis*)
M. abscessus
M. avium
M. chelonae
M. fortuitum
M. gastri
M. gordonae
M. intracellulare
M. kansasii
M. marinum
M. nonchromogenicum
M. peregrinum
M. scrofulaceum
M. simiae
M. szulgai
M. terrae
M. triviale
M. xenopi

been several cases of false positives. Attempts to differentiate *Mycobacterium ulcerans* subsp. *shinshuense* (the causative agent of Buruli ulcer [BU] in Japan)[9] from *M. ulcerans* using this kit yield an identification of *M. marinum* (Fig. 4).[19] *Mycobacterium massiliense* and *Mycobacterium bolletii* are misidentified as *Mycobacterium abscessus*,[20] while *Mycobacterium heckeshornense* is classified as *Mycobacterium xenopi*. Additional laboratory procedures are required to discriminate these species. Polymerase chain reaction (PCR) detection of insertion sequence (IS)*2404* can be used to differentiate *M. ulcerans* and/or *M. ulcerans* subsp. *shinshuense* from *M. marinum*.[9,19] However, combination sequence analysis using the *hsp65* and *rpoB* genes is required to separate *M. abscessus*, *M. massiliense* and *M. bolletii*.[20]

Gene amplification assays

Diagnostic genotyping kits for the detection of pathogenic mycobacterial genomes such as *M. tuberculosis*, *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium kansasii* are commercially available.[21–23] The correct results

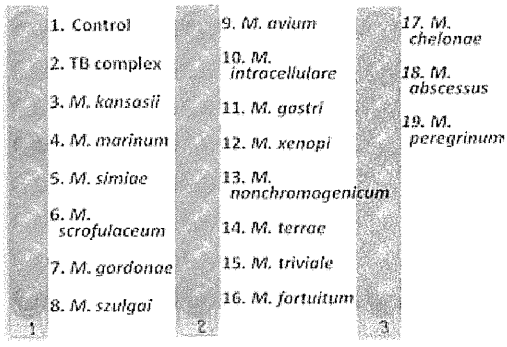

Figure 4. Commercially available DNA–DNA hybridization assay using an *Mycobacterium ulcerans* subsp. *shinshuense* clinical isolate. Blue color change was observed in a well of *M. marinum*.

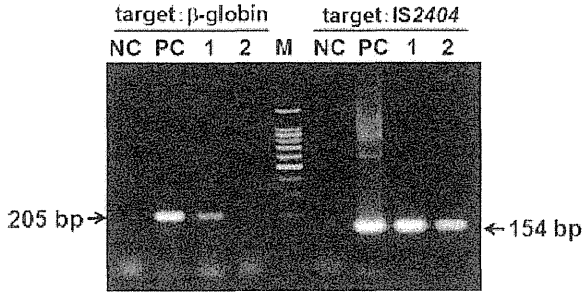

Figure 5. Gel electrophoresis of polymerase chain reaction products amplified using template DNA extracted from formalin-fixed and paraffin-embedded sections. 1, sample 1; 2, sample 2; NC, negative control; PC, positive control.

can be rapidly obtained if certain mycobacteria are present in the specimen material. However, when the results are negative, the smear test and/or culture assays should be performed for the other NTM.

Culturing an SGM can take several weeks. Therefore, genotyping assays such as species-specific PCR (Fig. 5) or 16S rRNA gene sequencing (described below) are extremely effective. Even for RGM species such as *Mycobacterium chelonae*, *M. abscessus* or *Mycobacterium fortuitum*, the primary isolation takes several weeks, but identification can be hastened using genotypic assays in parallel with culture assays. Specimens are sometimes processed into formalin-fixed paraffin-embedded blocks or frozen embedded blocks, making culture assays impossible. The sensitivity and specificity of PCR from these blocks is variable, depending on the condition of the DNA in the specimens. The order of preferred samples for genotypic analysis (from best to worst) is: cultured colonies > fresh specimens > ethanol-fixed specimens > formalin-fixed paraffin-embedded specimens.

Identification using the 16S rRNA gene sequence

If an abundance of bacterial DNA is available, sequence analysis of the first one-third of the 16S rRNA gene (Table 4, primer set; 8F16S-1047R16S) can be used for strain comparisons in the Ribosomal Differentiation of Medical Micro-organisms (RIDOM) database (www.ridom-rdna.de/).[29–31] RIDOM uses the sequence text to find the top 10 reference strains with the highest homology to the query sequence. The sequences contain approximately 500 bp, which includes hypervariable regions A and B of the mycobacterial 16S rRNA gene (*E. coli* positions 54–510). Sequence homology greater than 99% usually leads to a call that two strains are identical. However, this method cannot differentiate between *M. ulcerans* subsp. *shinshuense*, *M. marinum* and *M. ulcerans* due to their sequence similarity. In other cases, *M. kansasii* and *Mycobacterium gastri* exhibit 100% homology in the first 500 bp of their 16S rRNA genes, as do members of the *M. chelonae-abscessus* group.[32,33] These strains require additional methods for differentiation. Sequence analysis of the majority of the 16S rRNA gene (1500 bp) allows differentiation of *M. ulcerans* subsp. *shinshuense*, *M. marinum* and *M. ulcerans* (Table 5).[19,34] The longer sequence read also distinguishes *M. chelonae* from the rest of the *M. chelonae-abscessus* group. Still, even this methodology cannot differentiate between *M. abscessus*, *M. bolletii* and *M. massiliense*.[20] There are other databases such as the Ez Taxon identification service (EzTaxon-e) and the basic local alignment search tool (BLAST), but they have no quality control standards for the submission of reference sequences.[35,36]

Identification using other housekeeping gene sequences

The sequence of the 16S rRNA gene (first one-third) cannot identify or differentiate some mycobacterium strains, but the entire gene is relatively large, and large amounts of DNA template are required to obtain the entire sequence. A more sensitive method for the targeting of multiple housekeeping genes

Table 4. Primers used for NTM and *M. ulcerans* detection and identification

Primer	Sequence	Target and/or purpose (amplified fragment size)	Reference
8F16S 1047R16S 830F16S 1542R16S	5′-AGAGTTTGATCCTGGCTCAG-3′ (positions 8 to 27)[a] 5′-TGCACACAGGCCACAAGGGA-3′ (positions 1047 to 1028)[a] 5′-GTGTGGGTTTCCTTCCTTGG-3′ (positions 830 to 849)[a] 5′-AAGGAGGTGATCCAGCCGCA-3′ (positions 1542 to 1523)[a]	Mycobacterial 16S rRNA gene, PCR (1500 bp), sequencing	24
TB11 TB12	5′-ACCAACGATGGTGTGTCCAT-3′ 5′-CTTGTCGAACCGCATACCCT-3′	Mycobacterial *hsp65 gene*, PCR (441 bp), sequencing	25
MabrpoF MabrpoR	5′-GAGGGTCAGACCACGATGAC-3′ (positions 2112–2131)[b] 5′-AGCCGATCAGACCGATGTT-3′ (positions 2559–2541)[b]	Mycobacterial *rpoB gene*, PCR (449 bp), sequencing	20
MF MR	5′-CGACCACTTCGGCAACCG-3′ 5′-TCGATCGGGCACATCCGG-3′	Mycobacterial *rpoB gene*, PCR (341 bp), sequencing	26
ITSF ITSR	5′-TTGTACACACCGCCCGTC-3′ 5′-TCTCGATGCCAAGGCATCCACC-3′	Mycobacterial 16S-23S ITS region, PCR (340 bp), sequencing	27
PU4F PU7Rbio	5′-GCGCAGATCAACTTCGCGGT-3′ 5′-GCCCGATTGGTGCTCGGTCA-3′	*M. ulcerans* IS*2404*, PCR (154 bp)	28

[a]Nucleotide positions were assigned using the coli *E. coli* 16S rRNA gene sequence as a reference.[b]Primer design and nucleotide positions were based on the *M. tuberculosis rpoB* gene sequence (Genbank accession no. L27989). ITS, internal transcribed spacer; PCR, polymerase chain reaction.

Table 5. 16S rRNA gene sequences differentiating *Mycobacterium ulcerans* and related species[19]

Organism	Origin	492[a]	1247	1288	1449–1451
M. ulcerans subsp. *shinshuense* ATCC 33728	Japan	GGGGA	GTGCA	AAGGC	ACCC—TTTG
M. ulcerans subsp. *shinshuense* LRC 0501	Japan	GGGGA	GTGCA	AAGGC	ACCC—TTTG
M. ulcerans ITM 98–912	China	GGGGA	GTGCA	AAGGC	ACCC—TTTG
M. ulcerans Agy99	Ghana	GGAGA	GTGCA	AACGC	ACCCTTTTTG
M. ulcerans ATCC 19423^T	Australia	GGAGA	GTGCA	AACGC	ACCC—TTTG
M. ulcerans 1615	Malaysia	GGAGA	GTGCA	AACGC	ACCC—TTTG
M. ulcerans 5143	Mexico	GGAGA	GTGCA	AAAGC	ACCC—TTTG
M. marinum ATCC 927^T	USA	GGAGA	GTACA	AAAGC	ACCC—TTTG

[a]Nucleotide position(s) were based on *Escherichia coli* 16S rRNA gene sequence.
Underline indicated differieng residue(s).

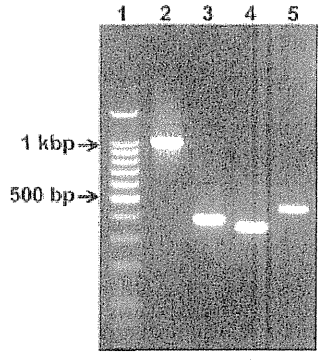

Figure 6. Gel electrophoresis of polymerase chain reaction products from skin biopsy specimens using a 2% agarose gel. Lane 1, 100-bp ladder; lane 2, 16S rRNA gene (8F16S-1047R16S); lane 3, internal transcribed spacer region; lane 4, *rpoB* gene (MF-MR); lane 5, *hsp65* gene.

for PCR and sequence analysis was required.[37] In addition to the 16S rRNA gene, we analyzed the DNA sequences of heat shock protein 65 (*hsp65*), *rpoB* and the 16S–23S intergenic spacer region (ITS region). Table 4 shows the sets of primers applicable to most strains of mycobacterium. Figure 6 shows the result of gel electrophoresis analysis after PCR using template DNA extracted from regions of affected skin and primers for the 16S rRNA gene (8F16S-1047R16S), the ITS region, *rpoB* (MF-MR) and *hsp65*. This figure shows amplified single bands; however, extra bands or inadequate amplification are sometimes apparent. The *rpoB* gene is the most polymorphic of the regions examined and is, therefore, very useful for identification, but the acquisition of PCR products is relatively difficult and the preparation of two different primer sets (MabrpoF-MabrpoR and MF-MR) is required to achieve the desired results. RIDOM database analysis using sequences of the ITS region and the 16S rRNA gene will find strains with higher levels of homology. In contrast, a BLAST search of *rpoB*

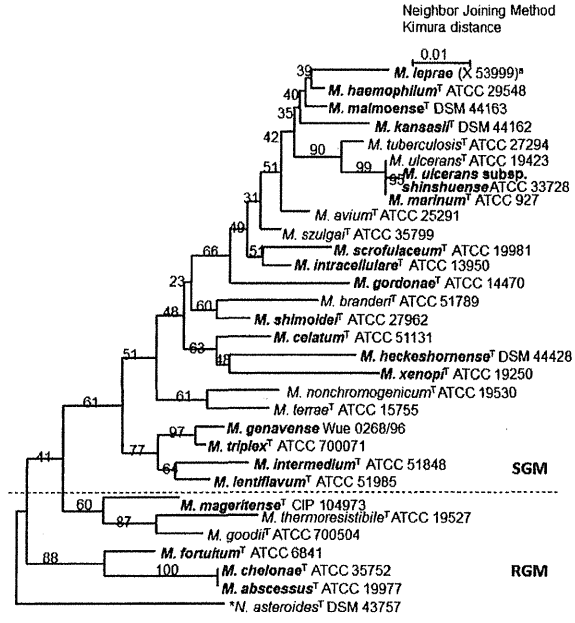

Figure 7. Phylogenetic trees of common pathogenic mycobacteria. A bold letter indicates the species which have been identified in our institute.

and *hsp65* gene would be needed for further consideration. Because quality control is not used in BLAST, care should be taken when it is used as a comparison tool. After the analysis of four different gene sequences, the most homologous bacteria can be chosen as phylogenetic mycobacteria. Sometimes, there is no match to the four different genes, which might be indicative of a novel species.[38] It is recommended that samples be sent to a specialized institution for accurate identification of rare strains.

Phylogenetic trees analysis

Approximately 30 species of mycobacteria have been reported as human pathogens in Japan, and it is assumed that these species cause disease throughout Asia as well. Figure 7 shows a phylogenetic tree of these 30 pathogens based on 16S rRNA sequence data. *M. leprae* is closely related to *Mycobacterium haemophilum*, while *M. ulcerans* subsp. *shinshuense* is a close relative of *M. ulcerans*, *M. marinum* and even *M. tuberculosis*, which are all only distantly related to the RGM species.

Drug susceptibility test

Antibiotic susceptibility profiles are key considerations in the choice of treatment options for mycobacterial infections. Cultured bacteria from cutaneous wounds can be used in susceptibility testing in order to choose antibiotics. The test is performed according to the microdilution method approved by the Clinical Laboratory and Standards Institute (CLSI).[39] In general, microdilution is an easy and reliable technique for this purpose. The approved method was revised recently to state that cation-adjusted Müller–Hinton broth (CAMHB) should be used as culture media for the assay. In particular, CAMHB without OADC supplementation should be used to determine the concentration of drugs against RGM isolates. Mutations in certain genes have been associated with antibiotic resistance (Table 6). Genotypic analysis of these genes can be performed,

Table 6. Drug resistance-related mutations in *Mycbacterium tuberculosis* and other mycobacteria

Drug	Related gene	Gene product
Rifampicin (RFP)	*rpoB*	DNA-dependent RNA polymerase β subunit[40]
Isoniazid (INH)	*katG*	Catalase-peroxidase[41]
	inhA	NADH-dependent enoylacyl carrier protein reductase[42]
	ahpC	Alkyl hydroxyperoxidase[43]
Ethambutol (EB)	*embB*, *embA*, *embC*	Arabinosyl transferase[44]
Pyrazinamide (PZA)	*pncA*	Pyrazinamidase/ nicotinamidase[45]
Streptomycin (SM)	*rpsL*	Ribosomal protein S12[46]
	*r*RNA	16S rRNA[47]
Kanamaycin (KM)	*r*RNA	16S rRNA[48]
Diaminodiphenyl sulfone (DDS, dapsone)	*folP*	Dihydropteroate synthase[49]
Fluoroquinolones	*gyrA*, *gyrB*	DNA gyrase A subunit, B subunit[50]
Clarithromycin (CAM)	*r*RNA	23S rRNA[51]

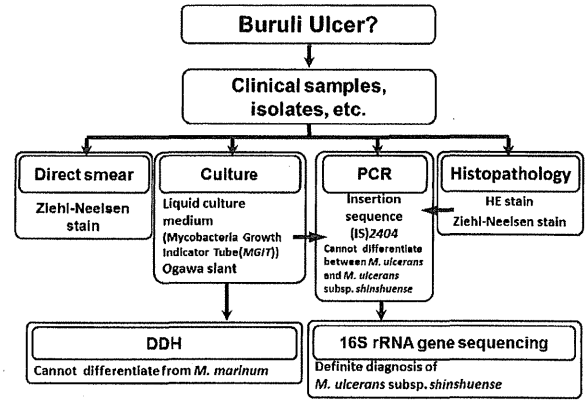

Figure 8. Flow chart for the differential identification of Buruli ulcer. DDA, DNA–DNA hybridization; HE, hematoxylin–eosin; PCR, polymerase chain reaction.

but the process could prove to be very labor intensive in a clinical setting.

CASE STUDY: IDENTIFICATION OF A CUTANEOUS NTM INFECTION

Diagnosis of BU[52–54]

A PCR assay targeting IS*2404* is frequently used to diagnose BU[28] The PCR-amplified sequences of the IS*2404* target are less than 200 bp (Table 4). At present, IS*2404* has been found only in two strains of human pathogenic mycobacteria: *M. ulcerans* and *M. ulcerans* subsp. *shinshuense*. Moreover, it is present at more than 200 copies/genome, so the sensitivity is extremely high and it is suitable for screening and differential identification.[55,56] It is noteworthy that some groups of mycobacterium derived from fish and amphibians also carry IS*2404*,[57–60] therefore, caution must be exercised during environmental studies. Figure 8 shows the flow chart for the diagnosis of BU. It is principally the same as that found in the World Health Organization manual for the diagnosis of *M. ulcerans* disease.[61]

Practical PCR assay using paraffin specimens

DNA should be fragmented when dealing with formalin-fixed paraffin-embedded clinical samples. The amplification of long DNA regions by PCR is often difficult, but assays targeting shorter regions of less than 200 bp are more feasible. The caveat is that detection sensitivity is very low with formalin-fixed paraffin-embedded samples; however, the transport and storage of specimens are relatively easy. In practice, the quality and integrity of DNA from these specimens should be confirmed. Amplification of the β-globin region of human genomic DNA should be used as a control. In Figure 5 (to the left of the marker), lanes 1 and 2 show positive signals for the human β-globin gene, demonstrating that the quality of DNA from the formalin-fixed paraffin-embedded specimens was sufficient for amplification. To the right of the marker lane are amplicons of IS*2404*. Lanes 1 and 2 show a 154-bp band, the right size of the IS*2404* target in *M. ulcerans* and *M. ulcerans* subsp. *shinshuense*. No signal was observed in the negative control (NC) lane, while 154-bp and 205-bp bands appeared In the positive control (PC) lane.

CONCLUSION

At present, 156 species and 13 subspecies of mycobacteria have been registered.[62] New mycobacterial species are being reported because liquid medium is broadly used as the isolation medium, and the ease of isolation from broth culture has increased significantly. Moreover, the progression of genotypic analysis has contributed to this increased rate of discovery. The increase in the number of NTM patients has highlighted the importance of rapid diagnosis of mycobacterial agents.

An elementary, but important, precautionary statement is that specimens should be collected before starting antibiotic treatment. For cases in which the mycobacterium species are rare or are difficult to diagnose, further analysis may be required by a specialized institution.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor and Welfare of Japan to Y. H., M. M. and N. I.; a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan for Y. H.; and a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science to K. N.

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Published October 24, 2012, doi:10.4049/jimmunol.1202479

Cutting Edge

THE JOURNAL OF IMMUNOLOGY

Cutting Edge: Nitric Oxide Inhibits the NLRP3 Inflammasome

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Although the NLRP3 inflammasome plays a pivotal role in host defense, its uncontrolled activation is associated with inflammatory disorders, suggesting that regulation of the inflammasome is important to prevent detrimental effects. Type I IFNs and long-term LPS stimulation were shown to negatively regulate NLRP3 activation. In this study, we found that endogenous NO is involved in the regulation of NLRP3 inflammasome activation by either IFN-β pretreatment or long-term LPS stimulation. Furthermore, *S*-nitroso-N-acetylpenicillamine (SNAP), an NO donor, markedly inhibited NLRP3 inflammasome activation, whereas the AIM2 and NLRC4 inflammasomes were only partially inhibited by SNAP. An increase in mitochondrial reactive oxygen species induced by ATP was only modestly affected by SNAP treatment. Interestingly, *S*-nitrosylation of NLRP3 was detected in macrophages treated with SNAP, and this modification may account for the NO-mediated mechanism controlling inflammasome activation. Taken together, these results revealed a novel role for NO in regulating the NLRP3 inflammasome. *The Journal of Immunology*, 2012, 189: 000–000.

The inflammasome is a multiprotein complex that mediates the activation of caspase-1, which then processes pro–IL-1β and pro–IL-18 into mature IL-1β and IL-18. Among a number of sensor proteins reported to be involved in inflammasome activation, NLRP3, NLRP1b, NLRC4, and AIM2, have been established as the major sensors for the recognition of various pathogens or damage-associated molecular patterns.

NLRP3 activation is triggered by different types of stimuli (e.g., whole pathogens, uric acid crystals, nigericin, ATP) (1). The precise mechanism of NLRP3 inflammasome activation has not been determined; however, NLRP3 agonist-induced generation of mitochondrial reactive oxygen species (ROS) is likely to play a major role in NLRP3 activation (2–5). Despite the fact that NLRP3 inflammasome contributes to host defense against microbial pathogens, excessive activation due to mutations in the NLRP3 gene has been associated with a spectrum of autoinflammatory disorders collectively known as "cryopirin-associated periodic syndromes" (1). Additionally, NLRP3 has been implicated in obesity-induced inflammation and insulin resistance (6). Thus, appropriate regulation of inflammasome activation appears to be important to avoid detrimental effects.

It was demonstrated that activation of the inflammasome is regulated by several mechanisms. For instance, the protein level of NLRP3 is relatively low in resting macrophages, so that NLRP3 inflammasome formation is hardly induced until the expression level is increased by exogenous and endogenous factors, including TLR agonists and proinflammatory cytokines. This suggests that activation of the NLRP3 inflammasome is limited under normal conditions (7). In contrast, long-term priming with the TLR4 agonist LPS results in the suppression of NLRP3 inflammasome activity, reportedly owing to the induction of TRIM30 (8). It was also demonstrated that type I IFNs negatively regulate the activation of caspase-1 induced by NLRP3 agonists but not that induced by AIM2 or NLRC4 agonists, although the mechanism remains unclear (9). In a report published before the establishment of the inflammasome concept, it was suggested that NO directly inhibits caspase-1 activity, thereby preventing the release of mature IL-1β and IL-18 in response to LPS and IFN-γ (10). Because type I IFNs also induce the expression of IFN regulatory factor 1, a transcription factor required for the expression of inducible NO synthase (iNOS) (11), we addressed whether NO is involved in the inhibition of the NLRP3 inflammasome by type I IFNs. In the current study, we analyzed the role of NO in the regulation of NLRP3 inflammasome activation.

Materials and Methods

Mice

Female C57BL/6 (wild-type [WT]) mice were purchased from Japan SLC. IFN-α/β receptor 1 (IFNAR1) knockout (KO) mice were kindly provided

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Received for publication September 5, 2012. Accepted for publication October 4, 2012.

This work was supported by Grants-in-Aid for Scientific Research (B) and (C) and a Grant-in-Aid for Young Scientists (B) from the Japan Society for the Promotion of Science.

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The online version of this article contains supplemental material.

Abbreviations used in this article: IFNAR1, IFN-α/β receptor 1; iNOS, inducible NO synthase; KO, knockout; L-NMMA, N^G-monomethyl-L-arginine; mtROS, mitochondrial reactive oxygen species; poly(dA:dT), poly(deoxyadenylic-thymidylic) acid; rIFN-β, recombinant mouse IFN-β; ROS, reactive oxygen species; SNAP, *S*-nitroso-*N*-acetylpenicillamine; WT, wild-type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1202479

by Prof. Michel Aguet (Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland). KO mice for iNOS were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in specific pathogen-free conditions and used at 7–9 wk of age. All animal experimental procedures were approved by the Animal Ethics and Research Committee of Kyoto University Graduate School of Medicine.

Reagents

Ultra-pure LPS, Pam_3CSK_4, and *Salmonella* flagellin were purchased from InvivoGen (San Diego, CA); ATP was purchased from Amersham Biosciences (Piscataway, NJ); nigericin, poly(deoxyadenylic-thymidylic) acid [poly(dA:dT)], *S*-nitroso-*N*-acetylpenicillamine (SNAP), 8-bromoguanosine 3′,5′-cyclic monophosphate sodium salt, S-methyl methanethiosulfonate, and sodium L-ascorbate were purchased from Sigma-Aldrich (St. Louis, MO); N^G-monomethyl-L-arginine (L-NMMA) was purchased from Dojindo (Kumamoto, Japan); EZ-Link Biotin-HPDP was purchased from Pierce (Rockford, IL); recombinant mouse IFN-β, carrier-free (rIFN-β) was purchased from PBL Interferon Source (Piscataway, NJ); Griess reagent kit was purchased from Invitrogen (Grand Island, NY); anti-NLRP3 mAb (Cryo-2) and anti-ASC pAb (AL177) were purchased from Enzo Life Sciences (Exeter, U.K.); anti–caspase-1 p10 (M-20) pAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-mouse IL-1β biotinylated Ab was purchased from R&D Systems (Minneapolis, MN); anti-iNOS mAb was purchased from Transduction Laboratories (Lexington, KY); ELISA kit for mouse IL-1β was purchased from eBioscience (San Diego, CA); and recombinant mouse IL-18, anti-mouse IL-18 capture Ab, and anti-mouse 1L-18 biotin-labeled Ab were purchased from Medical & Biological Laboratories (Nagoya, Japan). The following primers were used: TRIM30 sense: 5′-GGCACAGTCTTTGCTCTGCAGTGTG-3′, antisense: 5′-GCAGTTGCCCTTCCCGTCTG-3′ and β-actin sense: 5′-TGGAATCCTGTGGCATCCATGAAAC-3′, antisense: 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′.

Cells

Peritoneal exudate cells collected 4 d after i.p. injection of thioglycollate medium were incubated for 3 h in RPMI 1640 medium supplemented with 10% FCS at 37°C in 5% CO_2. Nonadherent cells were washed out, and adherent macrophages were primed with LPS or Pam_3CSK_4 at a final concentration of 50 ng/ml in Opti-MEM I (Invitrogen). Then, the cells were treated with 5 μM nigericin for 60 min, 5 mM ATP for 30 min, 600 ng poly (dA:dT) using Lipofectamine LTX and Plus Reagent (Invitrogen) for 60 min, or 175 ng flagellin using GenomeONE-NEO Transfection Reagent HVJ Envelope vector Kit (Ishihara Sangyo, Osaka, Japan) for 60 min. rIFN-β and L-NMMA were used at final concentrations of 100 U/ml and 500 μM, respectively.

Results and Discussion

NO is involved in the inhibitory effect of IFN-β on caspase-1 activation via the NLRP3 inflammasome

IFN-β was shown to inhibit the expression of pro–IL-1β and caspase-1 activation via NLRP3 inflammasome, but the mechanism remains unknown (9). To analyze whether NO generated upon IFN stimulation is responsible for the inhibitory effect of type I IFNs on NLRP3 inflammasome activation, peritoneal macrophages were primed with Pam_3CSK_4, a TLR2 ligand, in the presence or absence of rIFN-β and/or L-NMMA, an NO synthase inhibitor, before being stimulated with nigericin. In agreement with the previous study, we observed clear decreases in nigericin-induced caspase-1 activation, the protein level of pro–IL-1β, and the secretion of mature IL-1β when priming was done in the presence of rIFN-β (Fig. 1A, 1B). The specificity of the inhibitory effect of IFN-β was confirmed using IFNAR1-deficient macrophages (Fig. 1A, 1B). As expected, both the expression of iNOS and NO production were strongly induced in WT macrophages primed with Pam_3CSK_4 and rIFN-β (Fig. 1A, 1C). Interestingly, the addition of L-NMMA, which inhibited the production of NO, canceled the inhibitory effect of IFN-β on caspase-1 activation (Fig. 1A, 1C). In contrast, L-NMMA only modestly restored the level of pro–IL-1β in rIFN-β–treated macrophages

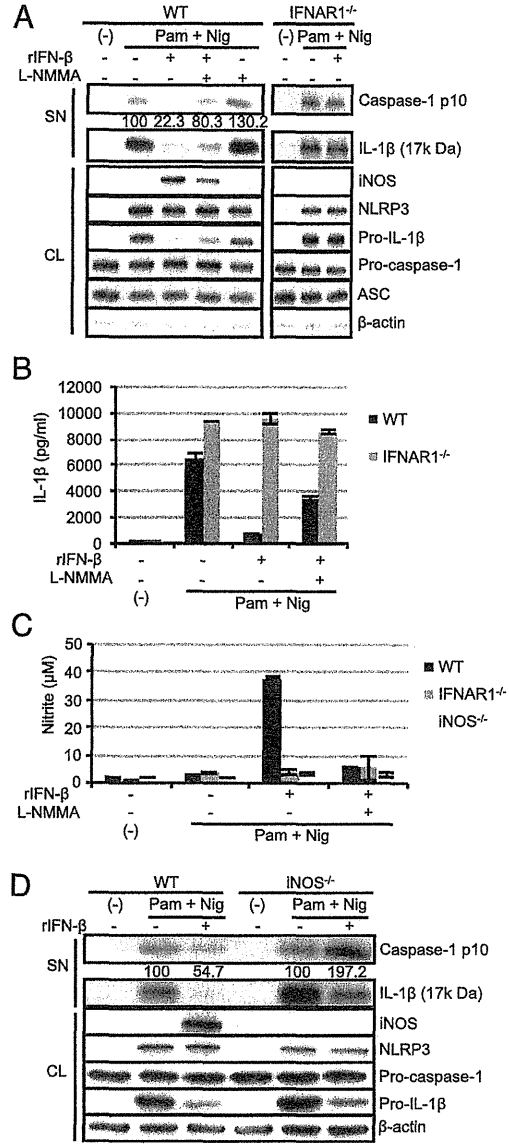

FIGURE 1. iNOS is required for inhibition of the NLRP3 inflammasome by IFN-β. Macrophages from WT mice (**A–D**), IFNAR1-deficient mice (A–C), or iNOS-deficient mice (C, D) were left unprimed or primed with Pam_3CSK_4 (Pam) in the presence or absence of rIFN-β and/or L-NMMA for 12 h. The cells were stimulated or not with nigericin (Nig) for 30 min. (A and D) Culture supernatants and cell lysates were collected and subjected to Western blotting with Abs specific to the indicated proteins. The numbers represent the relative intensities of the caspase-1 p10 fragment after normalization to control values (%). (B) The level of IL-1β in the culture supernatants was determined by ELISA. (C) The concentration of nitrite in the culture supernatants was measured using a Griess reagent kit. Experiments were repeated at least three times with consistent results. Data represent means and standard deviations of triplicate assays.

primed with Pam_3CSK_4, resulting in a partial recovery of the secretion of mature IL-1β induced by nigericin (Fig. 1A, 1B). These data suggested that NO is the molecule contributing to the inhibitory effect of IFN-β on NLRP3 inflammasome activation. In iNOS-deficient macrophages, NO production in response to Pam_3CSK_4 and rIFN-β was not observed, and rIFN-β treatment did not reduce the nigericin-induced caspase-1 activation (Fig. 1C, 1D). Thus, we confirmed that NO plays a critical role in the inhibition of the NLRP3 inflammasome by IFN-β. Recently, a study showed that IFN-β induces the

expression of SOCS1, which mediates the inhibition of ROS production (12). Therefore, our findings suggest a novel mechanism of inhibition of inflammasome activation by type I IFNs in addition to the SOCS1-mediated one.

NO donor SNAP inhibits the NLRP3 inflammasome, whereas the AIM2 and NLRC4 inflammasomes are only partially affected

To further confirm the inhibitory effect of NO on the activation of NLRP3 inflammasome, macrophages primed with Pam_3CSK_4 were incubated in the presence of SNAP, an NO donor, before stimulation with inflammasome activators. Substantial levels of NO were generated in the cultures when various concentrations (125–500 μM) of SNAP were added (Fig. 2A). Under this experimental condition, caspase-1 activation and the secretion of mature forms of IL-1β and IL-18 induced by nigericin or ATP were significantly decreased by SNAP in a dose-dependent manner (Fig. 2B–E). This result substantiated the NO-dependent inhibition of the NLRP3 inflammasome. In contrast, SNAP treatment partially diminished these cellular responses induced by poly(dA:dT) or flagellin, agonists for AIM2 and NLRC4, respectively (Fig. 2B–E). Judging from these results, it appeared that the NLRP3 inflammasome is more susceptible to NO than are the AIM2 and NLRC4 inflammasomes and that the inhibitory effect of NO on the NLRP3 inflammasome is not simply due to direct inhibition of caspase-1 enzymatic activity by NO, as proposed in a previous report (10).

Endogenous NO induced by long-time LPS priming serves as a negative regulator of NLRP3 inflammasome activation

It was shown that, after long-term priming with LPS, macrophages become refractory to the activation of NLRP3. Actually, macrophages primed with LPS for 12 h secreted markedly lower levels of IL-18 after stimulation with nigericin or ATP compared with those primed for 4 h (Fig. 3A). A possible explanation for this inhibitory effect is that LPS induces the expression of TRIM30, which negatively regulates the NLRP3 inflammasome (8). However, we observed that long-term priming with Pam_3CSK_4 did not lead to suppression of NLRP3 inflammasome activation, even though TRIM30 expression was upregulated to some extent (Fig. 3B, 3C). LPS, but not Pam_3CSK_4, induced type I IFN production, iNOS expression, and NO generation in macrophages (Fig. 1C, Supplemental Fig. 1A, 1B) (11, 13). Therefore, we hypothesized that NO induced by long-term LPS priming may mediate the inhibitory effect. To test this possibility, iNOS-deficient macrophages were stimulated with nigericin or ATP after being primed with LPS for 4 or 12 h. We found that these NLRP3 agonists induced higher levels of IL-18 secretion from iNOS-deficient macrophages than from WT macrophages if the macrophages were primed for 12 h (Fig. 3D). Similar results were obtained in experiments using L-NMMA (Supplemental Fig. 1C, 1D). In contrast, there was no significant difference between iNOS-deficient macrophages and WT macrophages in the secretion of IL-18 in response to NLRP3 agonists when the cells were primed with LPS for 4 h, at which time iNOS was not expressed (Fig. 3D, Supplemental Fig. 1E). Accordingly, our results suggest that endogenous NO induced by long-time LPS priming inhibits NLRP3 inflammasome activation. Secretion of IL-18 induced by flagellin or poly(dA:dT) after long-term priming with LPS was even greater than was that after a 4-h LPS priming (Fig. 3E), supporting that NLRC4 and AIM2 inflammasomes are more resistant to NO than is the NLRP3 inflammasome.

Analysis of the mechanism by which NO regulates NLRP3 inflammasome activation

To elucidate the mechanism by which NO exerts its inhibitory effect, particularly on the NLRP3 inflammasome, we measured mitochondrial ROS (mtROS), which are known to promote

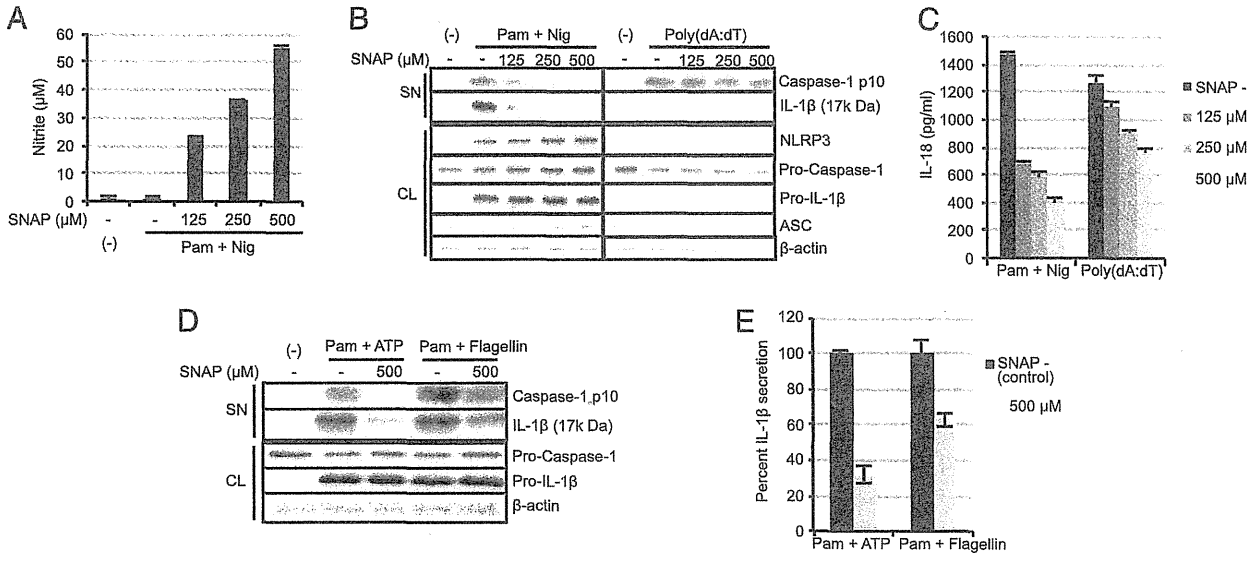

FIGURE 2. The NO donor SNAP inhibits the activation of the NLRP3 inflammasome. WT macrophages were primed with Pam_3CSK_4 (Pam) for 3 h and further treated or not with the indicated concentrations of SNAP for 1 h. The cells were then stimulated or not with nigericin (Nig) (**A–C**), ATP (**D**, **E**), or flagellin (D, E). (B and C) Unprimed cells were stimulated with poly(dA:dT) with or without SNAP pretreatment. (A) The concentration of nitrite in the culture supernatants was measured. (B and D) Culture supernatants and cell lysates were subjected to Western blotting with Abs specific to the indicated proteins. (C and E) The levels of IL-18 or IL-1β in the culture supernatants were determined by ELISA. (E) The percentage of IL-1β secretion was calculated as (IL-1β concentration in test sample)/(the average IL-1β concentration in control samples) × 100. Experiments were repeated at least two times with consistent results. Data represent means and standard deviations of triplicate assays.

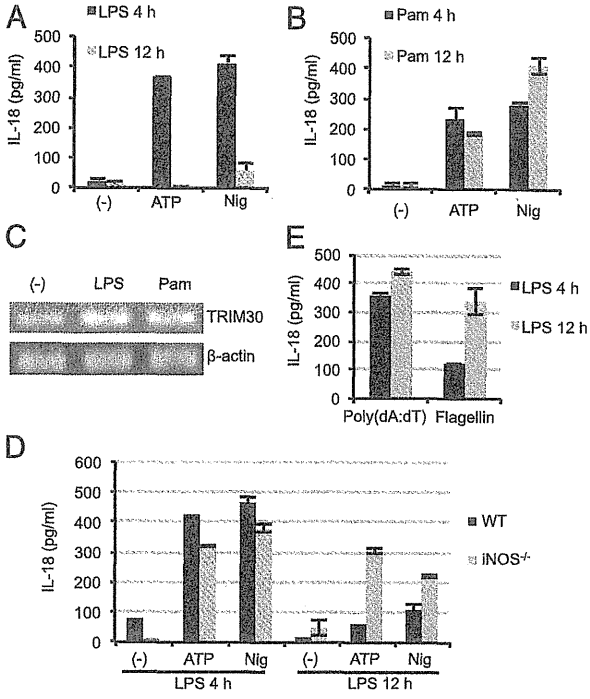

FIGURE 3. Long-term LPS priming suppresses NLRP3 inflammasome activation via induction of NO generation. Macrophages from WT mice or iNOS-deficient mice (**D**) were primed with LPS (**A**, D, **E**) or Pam_3CSK_4 (**B**) for 4 or 12 h. The cells were then stimulated or not with nigericin or ATP (A, B, D) or poly(dA:dT) or flagellin (E). The level of IL-18 in the culture supernatants was determined by ELISA. (**C**) Macrophages were stimulated with LPS or Pam_3CSK_4 for 12 h, and the expression of TRIM30 mRNA was analyzed by RT-PCR. Experiments were repeated at least two times with consistent results. Data represent means and standard deviations of triplicate assays.

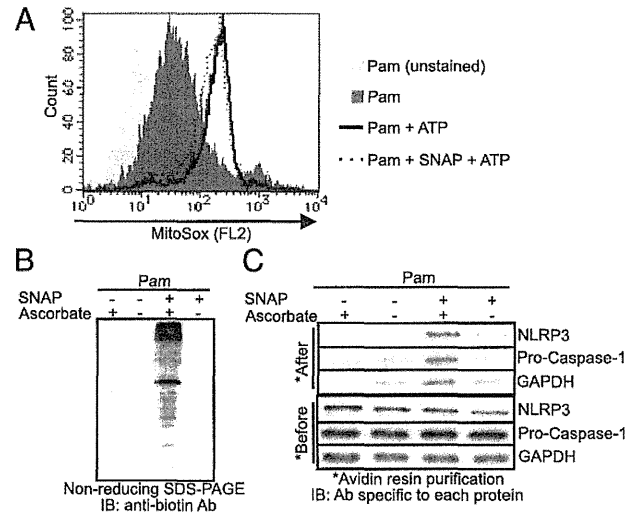

FIGURE 4. Analysis of the mechanism by which NO regulates the NLRP3 inflammasome. (**A–C**) WT macrophages were primed with Pam_3CSK_4 for 3 h and treated or not with SNAP (0.5 mM) for 1 h. (A) The cells were stimulated with ATP for 15 min, stained with MitoSOX (2.5 μM) for 15 min, and analyzed on a flow cytometer. (B and C) The cells were lysed, and free thiols were blocked with *S*-methyl methanethiosulfonate (20 mM). The lysates were reduced or not with sodium ascorbate (10 mM) and labeled with 200 μM of biotin-HPDP. (B) The labeled lysates were subjected to nonreducing SDS-PAGE and Western blotting with an anti-biotin Ab. (C) NLRP3, caspase-1, and GAPDH (positive control) were detected by Western blotting in the labeled lysates (*lower panels*) and in biotinylated protein fractions enriched from the same samples (*upper panels*). Experiments were repeated at least three times with consistent results.

NLRP3 activation (3–5). SNAP treatment only modestly affected the increase in mtROS induced by ATP (Fig. 4A), and it was difficult to conclude whether the inhibition of NLRP3 by NO depends solely on the decrease in mtROS generation. NO induces the upregulation of antioxidant genes, including thioredoxin reductase-1, which may disturb NLRP3 inflammasome activation (2) (Supplemental Fig. 2A). However, inhibition of NLRP3 inflammasome by just a 1-h treatment with SNAP was also observed in the presence of cycloheximide (Supplemental Fig. 2B, 2C). Thus, the de novo synthesis of antioxidant proteins did not appear to account for the inhibitory effect of NO on NLRP3 inflammasome, at least at an early time after exposure to SNAP. NO is known to increase the synthesis of the second messenger cyclic GMP, but IL-1β secretion was not affected by 8-bromoguanosine 3′,5′-cyclic monophosphate sodium salt, an analog of cyclic GMP (Supplemental Fig. 2D). *S*-nitrosylation is a covalent addition of an NO group onto protein cysteine thiols to form *S*-nitrosoproteins, and it has been increasingly recognized as a post-translational modification regulating the protein functions (14). We used the biotin-switch technique, as previously described, to determine whether the NLRP3 protein can be a target of *S*-nitrosylation (15). Biotinylated proteins were detected in cell lysates from macrophages treated with SNAP for 1 h, only when the lysates were reduced with ascorbate before labeling of protein cysteine thiols with biotin (Fig. 4B), indicating that *S*-nitrosoproteins in the lysates were successfully biotinylated. Interestingly, caspase-1 and NLRP3 were detected in *S*-nitrosylated proteins enriched from the labeled lysates using an avidin resin (Fig. 4C). We also found that the C-terminal region of NLRP3 is more susceptible to *S*-nitrosylation than is the N-terminal region (Supplemental Fig. 2E). From these results, we propose that a direct modification of the NLRP3 protein by *S*-nitrosylation is the mechanism responsible for the early inhibition of NLRP3 inflammasome activation by NO. A direct *S*-nitrosylation of caspase-1 itself may explain the partial inhibition of the AIM2 and NLRC4 inflammasomes by NO. Further analysis is necessary to confirm the relevance of *S*-nitrosylation as a regulatory mechanism of the NLRP3 inflammasome. Another possibility to be addressed is that NO interferes with other proteins involved in NLRP3 inflammasome activation or with mitochondrial DNA release, thereby inhibiting the NLRP3 inflammasome (4, 5).

In this study, we found that NO regulates the activation of NLRP3 inflammasome. Because both NO and NLRP3 are known to play roles in a wide range of physiological responses, NO-mediated inhibition of the NLRP3 pathway might have significance not only in the beneficial host defense against microbial pathogens but also in the pathophysiology of NLRP3-associated detrimental diseases. For example, NLRP3 was implicated in the pathogenesis of ischemia–reperfusion injury (16), and the involvement of NO was demonstrated in preconditioning-induced tissue protection from ischemia–reperfusion injury (17). Therefore, it is worth considering whether inhibition of NLRP3 activation by NO is at least one of the mechanisms of tissue protection by ischemic preconditioning. Further studies are necessary to fully describe the role and mechanism of the inhibitory effect of NO on NLRP3, and a better understanding of the mechanism may shed new light on the activation process of the NLRP3 inflammasome.

Acknowledgments

We thank Prof. Michel Aguet for permission to use the IFNRA1 KO mice and Prof. Jürg Tschopp, University of Lausanne, Epalinges, Switzerland, for permission to use the NLRP3 KO mice. We thank Prof. Shigekazu Nagata, Kyoto University, for providing the IFNRA1 KO mice and Prof. Hiroko Tsutsui, Hyogo College of Medicine, Hyogo, Japan, for providing the NLRP3$^{-/-}$ mice.

Disclosures

The authors have no financial conflicts of interest.

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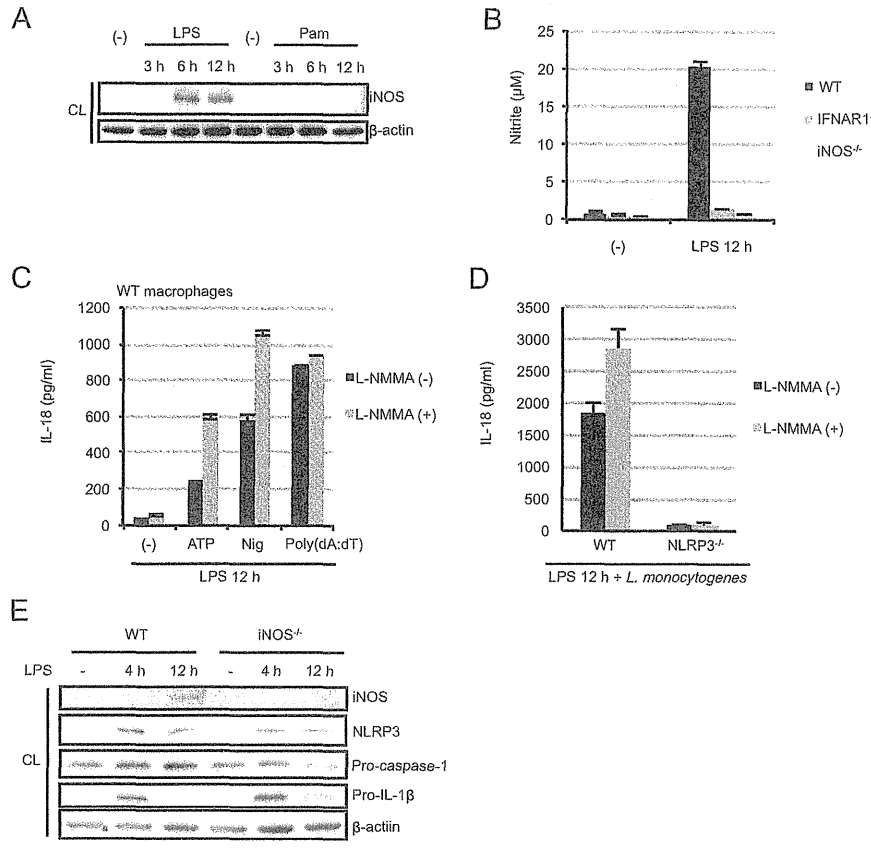
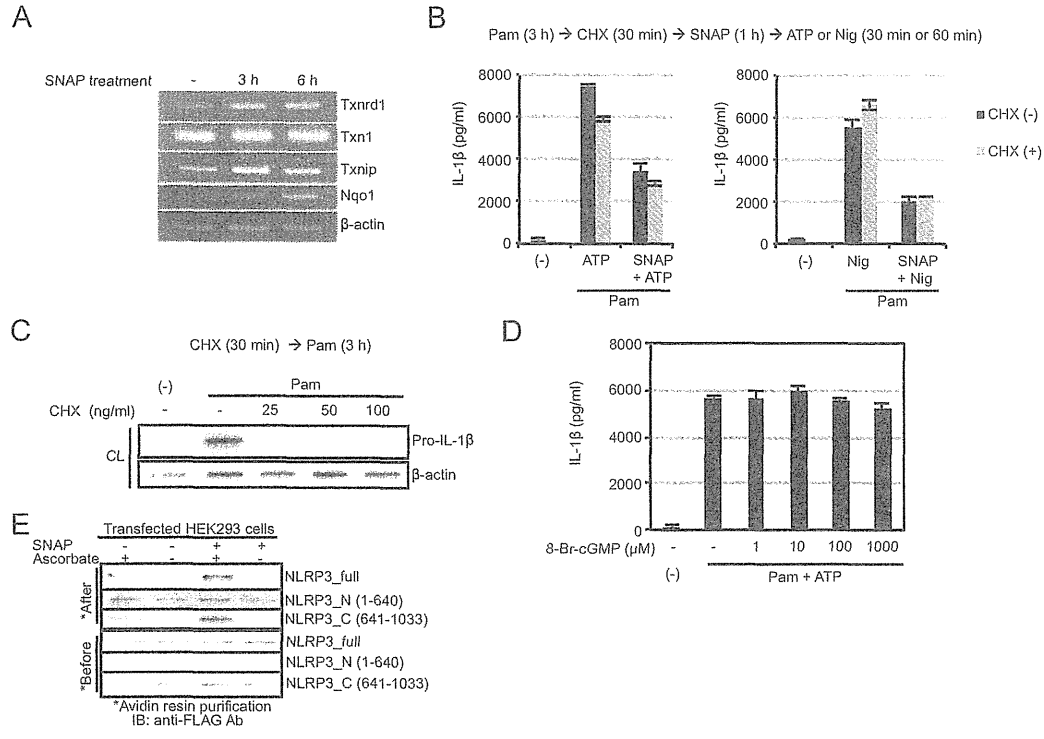
Supplemental Figure 1



Supplemental Figure 1: LPS, but not Pam_3CSK_4, induces iNOS expression. (A) Macrophages were stimulated with LPS or Pam_3CSK_4 for the indicated time periods, and the protein level of iNOS in cell lysates was analyzed by Western blotting with an anti-iNOS Ab. (B, E) Macrophages from WT mice, IFNAR1-deficient mice, or iNOS-deficient mice were stimulated with LPS for 4 or 12 h. (B) The concentration of nitrite in the culture supernatants was measured. (E) Cell lysates were subjected to Western blotting with antibodies specific to the indicated proteins. (C, D) WT or NLRP3-deficient macrophages were primed with LPS for 12 h in the absence or presence of L-NMMA. The cells were then stimulated with nigericin, ATP, or Poly(dA:dT) (C), or infected with live *Listeria monocytogenes* EGD at a multiplicity of infection (MOI) of 50 for 2 h (D), and the level of IL-18 in the culture supernatants was determined by ELISA. L-NMMA treatment significantly increased the secretion of IL-18 in response to nigericin, ATP, or *L. monocytogenes*. Although the induction of caspase-1 activation by *L. monocytogenes* infection at low MOIs has been reported to depend on multiple receptors, such as AIM2, NLRP3, and NLRC4, NLRP3 is entirely responsible for caspase-1 activation induced by infection with *L. monocytogenes* at an MOI of 50 after LPS priming (*Nature* 2006 **440**:228, *J. Immunol.* 2010 **185**:1186, and Supplemental Fig. 1D). Thus, the result suggested that the inhibition of the NLRP3 inflammasome by NO can occur when activated macrophages producing NO are infected with high doses of *L. monocytogenes*. NLRP3-deficient mice were kindly provided by Prof. Jürg Tschopp (University of Lausanne).

Supplemental Figure 2



Supplemental Figure 2: SNAP treatment resulted in the up-regulation of antioxidant genes, whereas de novo synthesis of proteins was not required for the early inhibition of the NLRP3 inflammasome by NO. (A) WT macrophages were incubated with or without SNAP for 3 or 6 h, and mRNA levels of thioredoxin reductase-1 (Txnrd1), thioredoxin 1 (Txn1), thioredoxin-interacting protein (Txnip), and NAD(P)H dehydrogenase [quinone] 1 (Nqo1) were analyzed by RT-PCR. The primers used are as follows; Txnrd1 sense: 5'-TGCTGGCTCAGAGGCTGTAT, antisense: 5'-CTTCGACCTGCCACCTCCTA; Txn1 sense: 5'-ATCTGGTTCTGCTGAGACGC, antisense: 5'-TGGAAGGTCGGCATGCATTT; Txnip sense: 5'-TTGAACCCACTCGGCTCAATCATGGTG, antisense: 5'-GACTGAGTCTCTGAAACACTGGAGTTC; Nqo1 sense: 5'-TTCTCTGGCCGATTCAGAGT, antisense: 5'-GGCTGCTTGGAGCAAAATAG. (B) WT macrophages were left unprimed or primed with Pam_3CSK_4 for 3 h and treated with or without 50 ng/ml of cycloheximide for 30 min. SNAP was then added to some wells. The cells were further incubated for 1 h and stimulated with ATP or nigericin for 30 min. (D) Macrophages primed with Pam3CSK4 were treated for 1 h with increasing concentrations of 8-Br-cGMP before stimulation with ATP for 30 min. (B and D) The level of IL-18 in the culture supernatants was determined by ELISA. (C) Macrophages were treated with the indicated concentrations of cycloheximide for 30 min and then stimulated with Pam_3CSK_4 for 3 h. The protein level of pro-IL-1β in the cell lysates was analyzed by Western blotting with an anti-IL-1β antibody. We verified that these concentrations of cycloheximide were sufficient to inhibit protein synthesis. (E) HEK293 cells were transiently transfected with FLAG-tagged expression vectors for full-length mouse NLRP3 (NLRP3_full) or its N-terminal (NLRP3_N) or C-terminal (NLRP3_C) fragments. After 48 h, the cells were treated with or without SNAP for 1 h, and S-nitrosylated proteins were labeled by the biotin switch method as mentioned in Fig. 4. The labeled lysates and biotinylated protein fractions were subjected to Western blotting with an anti-FLAG antibody. Experiments were repeated at least three times with consistent results. The expression vectors for NLRP3_full, NLRP3_N, and NLRP3_C were constructed in this study. Briefly, cDNAs encoding full-length mouse NLRP3 and its truncated fragments were amplified from a mouse spleen cDNA preparation by PCR using KOD Plus polymerase (TOYOBO) and primer sets indicated below. The amplified fragments were digested with restriction enzymes and inserted into the pFLAG-CMV2 vector (Sigma-Aldrich). The primers used are as follows; NLRP3_full sense: 5'-CCTGCGGCCGCAACGAGTGTCCGTTGCAAG, antisense: 5'-CCTGGTACCCTACCAGGAAATCTCGAAGACTA; NLRP3_N sense: 5'-CCTGCGGCCGCAACGAGTGTCCGTTGCAAG, antisense: 5'-TGGCAGTCGACTAAAAGTCTTCCTCCTGCATCTCG; NLRP3_C sense: 5'-AGGAGGCGGCCGCGGTGCAGAGTGCCATGGACCACT, antisense: 5'-CCTGGTACCCTACCAGGAAATCTCGAAGACTA.