Research Article
Toll-Like Receptor 4 Exacerbates Mycoplasma pneumoniae via Promoting Transcription Factor EB-Mediated Autophagy

Yan Liu, Jing Li, Xianfeng Lu, Shuangping Zhen, and Jing Huo

Pediatrics Department, Shanxi Provincial People's Hospital, Taiyuan, Shanxi 030014, China

Correspondence should be addressed to Yan Liu; 2020151232@stu.cpu.edu.cn

Received 20 May 2022; Revised 15 June 2022; Accepted 23 June 2022; Published 31 July 2022

Academic Editor: Mohammad Farukh Hashmi

Copyright © 2022 Yan Liu et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Mycoplasma pneumoniae (M. pneumoniae) is the most common cause of community-acquired pneumonia. Toll-like receptors (TLRs) play an essential role in pneumonia. The purpose of this study was to investigate the roles of TLR4 in M. pneumoniae. Mice were administrated with 100 μl (1 × 107 ccu/ml) of M. pneumoniae. HE staining was applied for histological analysis. The protein expression was determined by western blot. The cytokine level was detected by ELISA. The results showed that TLR4-deficient mice were protected from M. pneumoniae. However, downregulation of TLR4 inhibited inflammatory response and autophagy. Moreover, transcription factor EB (TFEB) participated in M. pneumoniae-induced inflammatory response and autophagy, while knockdown of TLR4 downregulated TFEB and its nuclear translocation.

1. Introduction
Mycoplasma pneumoniae (M. pneumoniae) is an essential cause of community-acquired pneumonia [1]. The outbreaks of M. pneumoniae promote the progression of chronic and acute airway diseases, such as asthma [2]. Despite its self-limited and benign properties, M. pneumoniae is a public concern for the emerging antibiotic resistance [3]. Moreover, the outstanding side effects of M. pneumoniae therapies, such as fever, offset clinical results for its association with various complications (cytotoxicity, membrane fusion damage, invasive damage, toxic damage, DNA damage, autophagy, and inflammation) [4]. Therefore, to investigate an efficient therapy for M. pneumoniae is of vital importance.

Mycoplasmas induce the release of membrane-bound lipoproteins, which promotes the survival of bacteria [5]. Moreover, bacterial lipoproteins function as pathogenic substances to activate inflammatory responses via their recognition receptors [6]. For instance, M. pneumoniae increases the release of lipoproteins, which promotes inflammatory response via activating toll-like receptors (TLRs) [7]. TLRs are central pattern recognition receptors, which collectively participate in early recognition and the global and local immune response of the host to invading microbes [8]. TLRs recognize bacterial components, such as lipopolysaccharide (LPS), fibroblast-stimulating lipopeptide-1 (FSL-1), lipoarabinomannan, and zymosan, which increases the susceptibility of human hosts to various respiratory diseases [9–11]. TLR4, as a member of TLRs, plays a critical role in modulating immune responses to various molecular structures of microbes [12]. LPS-induced upregulation of TLR4 promotes M. pneumoniae infection [13]. Moreover, the activation of TLR4 signaling stimulates immune response and autophagy and exacerbates M. pneumoniae [14]. However, the potential roles of TLR4 in M. pneumoniae have not been fully elucidated.

In this study, we investigated the potential roles of TLR4 in M. pneumoniae and the underlying mechanisms. M. pneumoniae-induced overexpression of TLR4 contributed to inflammation response and promoted the transcriptional activity of TFEB, which further promoted lipid-induced autophagosome formation and evoked a proinflammatory response. Hence, the TLR4/TFEB axis may form a feedback loop to promote the release of proinflammatory cytokines.

2. Materials and Methods
2.1. M. pneumoniae Culture. M. pneumoniae strain M129 was consecutively cultured in Hayflick medium containing PPLO broth, horse serum (25%), and penicillin G for 7 days.
at 37°C. The concentration of M. pneumoniae was determined by 10² color change units (CCUs)/ml.

2.2. Animals. Wild-type and TLR4-/- mice (C57BL/10ScNJ, 14–17 g, 3 weeks old) were obtained from the Laboratory Animals Center of Shanxi Medical University. All mice were maintained under the following conditions: 23 ± 2°C, 50 ± 10% humidity, and 12 h light-dark cycles, specific pathogen-free condition for a week, and free access to food and water. Mice were randomly divided into the control group (n = 5, administrated with 100 µl normal saline). In M. pneumoniae group (n = 5), mice were intranasally infected with 100 µl of M. pneumoniae solution (1 x 10⁷ CCU/ml) once per day for two days as previously described [15]. Two days later, mice were anesthetized with chloroform and sacrificed with cervical decapitation.

This study was approved by the Animal Care Board of Shanxi Provincial People’s Hospital (SXPPH [2019]035).

2.3. HE Staining. The lower lobe of the right lung was fixed in 4% formaldehyde. Then, the sections were embedded with paraffin, dewaxed, dehydrated, and cultured with hematoxylin and eosin. Subsequently, sections were captured using Olympus microscopy (BX41-DP72) (×20).

2.4. Cell Treatment and Infection. Peritoneal macrophages RAW264.7 were exposed to 100 ng/ml LPS, 20 µg TAK-242 (a TLR4 inhibitor, MCE, Shanghai), 30 µM CCI-779 (a TFEB inhibitor, MCE, Shanghai), 40 µg chloroquine (CQ, Sigma-Aldrich, Shanghai), and 5 mM 3-methyladenine (3-MA, an autophagy inhibitor; Sigma-Aldrich). Next, the cells were infected with 0, 10, and 30 CFU/ml of M. pneumoniae for 24 h (OD595 = 0.1). The supernatants were harvested.

2.5. Enzyme-Linked Immunosorbert Assay (ELISA). The levels of proinflammatory cytokines, such as TGF-α and IL-1β, were detected by ELISA kits (R&D, USA). Briefly, cells were plated into a 96-well plate (2 x 10⁵ cells/well) and incubated with biotinylated antibodies. Subsequently, the levels of TGF-α and IL-1β were determined with a microplate reader (ELx808, BioTeck, Shanghai) at the wavelength of 450 nm.

2.6. Cell Transfection. Small interference RNA TLR4 (5'-GGACCTCTCTCAATGTCAA-3') and its negative control (5'-ACUACUGAGUCAGCAGUGA-3') were provided by GenePharma, Shanghai. Cells were seeded into a 24-well plate (2 x 10⁵ cells/well) and were transfected with si-TLR4 and its negative control by using Lipofectamine® 2000 (Invitrogen, USA) according to the manufacturer’s protocols for 48 h.

2.7. Western Blot. Total protein was collected from cells. Protein concentration was quantified using a BCA kit (Abcam, Shanghai). Afterwards, 30 µg of protein was separated by 12% SDS-PAGE at 120 V for 1 h. The separated protein was moved onto PVDF membranes (Millipore, Beijing), which was then sealed with nonfat milk. The membranes were incubated with primary antibodies including anti-TLR4 (ab13556, 1:500, Abcam, Shanghai), anti-TFEB (ab270604, 1:1000, Abcam, Shanghai), anti-LC3 I/II (ab128025, 1:1000, Abcam, Shanghai), anti-p62 (ab109012, 1:10000, Abcam, Shanghai), and anti-GAPDH (ab9485, 1: 2500, Abcam, USA) at 4°C overnight and with secondary antibodies (ab205718, 1:10000, Abcam, Shanghai) at room temperature for 2 h. The bands were captured with an ECL kit (Abcam, Shanghai) and quantified using Scion Image v. 4.0.2 software (Scion Corporation).

2.8. Immunofluorescence Assay. Cells were seeded into a 24-well plate (2 x 10⁵ cells/plate). Then, cells were fixed and permeabilized. Cells were blocked with 2% bovine serum albumin in PBS for 30 min. After incubated with primary antibodies against TFEB (ab128025, 1:1000, Abcam, Shanghai) and LC3 puncta (ab192890, 1:1000, Abcam, Shanghai), cells were cultured with DAPI. Subsequently, the results were visualized with a confocal microscope (Zeiss, Germany).

2.9. Statistical Analysis. Data were analyzed with SPSS 19.0 and expressed as mean ± SD. The difference among multigroups was analyzed by ANOVA followed by Duncan’s post hoc test. P < 0.05 was considered statistically significant.

3. Results

3.1. TLR4-Deficient Mice Were Protected from M. pneumoniae-Induced Inflammatory Damage of Lung Tissues. As shown in Figures 1(a) and 1(b), lung structure was without any obvious lesion in TLR4-deficient mice. In M. pneumoniae-infected mice, the alveolar walls were thickened. Only a few bronchial tubes were narrowed, suggesting that TLR4 deficiency relieved inflammatory lung tissue damage after M. pneumoniae infection.

3.2. M. pneumoniae Induced the Secretion of IL-1β and TNF-α from Macrophages. M. pneumoniae promotes the inflammation response [7]. Macrophages were stimulated by LPS. ELISA was conducted to determine the levels of proinflammatory cytokines, including TNF-α and IL-1β. As shown in Figure 2(a), the level of TNF-α was significantly increased in cells exposed to LPS. Moreover, M. pneumoniae increased the level of TNF-α in a dose-dependent manner. This was paralleled with IL-1β (Figure 2(b)).

3.3. TLR4 Was Required for M. pneumoniae-Induced Inflammation in Macrophages. TLR4 is collectively involved in the progression of M. pneumoniae [13, 14]. As shown in Figures 3(a) and 3(b), M. pneumoniae infection further increased the expression of TLR4. To further verify this, cells were treated with si-TLR4. As shown in Figures 3(c) and 3(d), the protein expression of TLR4 was significantly decreased in cells transfected with si-TLR4, suggesting that...
cells were significantly transfected. Knockdown of TLR4 significantly inhibited the secretion of TNF-α and IL-1β (Figure 3(e)).

3.4. The Activation of TLR4 Promoted M. pneumoniae-Induced Inflammatory Response. Autophagy is reported to contribute to M. pneumoniae [4]. To further investigate the roles of autophagy in M. pneumoniae, immunofluorescence and western blot assays were applied to determine the expression of autophagic markers. As shown in Figure 4(a), the expression of LC3 puncta was significantly increased in cells treated with M. pneumoniae, which was alleviated by TAK-242. Moreover, M. pneumoniae upregulated LC3 II, Beclin-1, and Atg5 in a dose-dependent manner (Figures 4(b) and 4(c)). CQ treatment was to exclude the possibility that the upregulation of LC3 II was the result of LC3 II-induced lysosomal dysfunction. The ratios of LC3 II/I were decreased by M. pneumoniae, which was reversed by CQ (Figure 4(d)). However, M. pneumoniae-induced downregulation of p62 was alleviated by CQ (Figure 4(e)).

3.5. M. pneumoniae-Induced the Release of Proinflammatory Cytokines via Activating TFEB. TLR4 interacted with TFEB to modulate inflammation and autophagy [16, 17]. Therefore, we further investigated the potential roles of TFEB in M. pneumoniae-induced inflammation response, and macrophages were activated by M. pneumoniae. Immunofluorescence and western blot were conducted to determine the expression of TFEB. As shown in Figure 5(a), M. pneumoniae promoted the translocation of TFEB from the cytosol to the nucleus. Similarly, M. pneumoniae significantly upregulated TFEB, which was reversed by TAK-242 (Figures 5(b) and 5(c)). Moreover, M. pneumoniae-induced release of TNF-α and IL-1β was abated by TFEB inhibitor CCI-779 (Figures 5(d) and 5(e)).

Our work is to probe the impact of atorvastatin on rats during the retention stage after orthodontic tooth movement and its associated molecular mechanism, and to provide a theoretical basis and potential treatment for the relevant research and clinical treatment of orthodontic tooth retention and recovery.
Figure 3: TLR4 promoted *M. pneumoniae*-induced proinflammation. (a) The protein expression of TLR4 detected by western blot. *M. pneumoniae* increased the protein of TLR4. (b) Quantification of A. (c) The protein expression of TLR4 detected by western blot. TLR4 was downregulated by si-TLR4. (d) Quantification of C. (e) The levels of IL-1β and TNF-α. Knockdown of TLR4 decreased the levels of IL-1β and TNF-α. **P < 0.01, ##P < 0.01.

Figure 4: Continued.
Figure 4: Autophagy promoted *M. pneumoniae*-induced inflammatory response. (a) The expression of LC3 II detected by immunofluorescence. Knockdown of TLR4 suppressed the upregulation of LC3 puncta induced by *M. pneumoniae*. (b) Quantification of A. (c) The protein expression of LC3 II/I, Beclin-1, and ULK1 determined by western blot. The protein expression of LC3 II/I and p62 increased the protein expression of LC3 II/I, Beclin-1, and ULK1. (d) Quantification of C. (e) The protein expression of LC3 II/I and p62 measured by western blot. *M. pneumoniae* increased the expression of LC3 II/I and p62, which was modulated by CQ. (f, g) Quantification of E. *P* < 0.01, ## *P* < 0.01.

Figure 5: Continued.
3.6. TFEB Enhanced M. pneumoniae-Induced Autophagy. To further investigate the underlying molecular mechanisms in M. pneumoniae-induced autophagy, we investigate the potential roles. As shown in Figure 6(a), 3-MA alleviated the effects of M. pneumoniae on the translocation of TEBF from the nucleus was antagonized by 3-MA. Moreover, TFEB antagonist CCI-779 abated the effects of M. pneumoniae on the expression of LC3 puncta (Figure 6(b)–6(d)).

4. Discussion

Pulmonary lesions and metabolic disorders contribute to immune response and oxidative stress [4]. In this study, M. pneumoniae upregulated TLR4 and induced an inflammatory response. Downregulation of TLR4 inhibited inflammatory response and autophagy. Hence, TLR4 may play a crucial role in modulating autophagy and inflammatory response in M. pneumoniae.

TLRs recognize invading M. pneumoniae through interacting with its ligands such as LPS, FSL-1, lipooarabinomannan, and zymosan [9–11]. TLR4, an essential member of the TLRs family, promotes the development of M. pneumoniae via driving the secretion of lipid-associated membrane proteins, pulmonary inflammatory factors, cell apoptosis, and autophagy [18]. However, the potential roles of TLR4 in M. pneumoniae are alluring. LPS-activated TLR4 plays little role in M. pneumoniae recognition. However, Luo et al. reveal that activation induces inflammatory response and autophagy in M. pneumoniae [19].

TFEB, as a crucial member of MiT/TFE family, is a master of lysosomal-related processes including lysosomal exocytosis and autophagy [20]. Moreover, TFEB is an important player in innate and adaptive immunity [21]. The activation of TFEB in macrophages exposed to bacteria and various toll-like receptor (TLR) ligands contribute to innate immune response and pathogen resistance and the increase in autophagy that is an essential factor of HIV replication [22,23]. Moreover, the translocation of TEBF to nucleus promotes SARS 3a induced autophagy and necrotic cell death [24]. Hence, to inhibit lysosomal-related processes via suppressing the expression of TLR4/TFEB signaling may be an efficient therapy for the emerging innate immune response and pathogen resistance in M. pneumoniae.

Autophagy plays a crucial role in immune response induced by pathogenic micro-organisms [25]. The exacerbation of contributes to impairment of airway epithelial barrier and autophagy [4]. The activation of TLR4 pathways induces autophagy after M. pneumoniae infection [14]. In this study, knockdown of TLR4 suppressed the autophagy of RAW264.7. Moreover, chloroquine increased the expression of the autophagic adaptor protein expression of p62 and LC3 II, excluding possibility of decreased lysosomal fusion and degradation. Moreover, TFEB is the master regulator of autophagy signaling and links autophagy to lysosomal biogenesis [26]. Overexpression of TFEB predicts poor prognosis of non-small cell lung cancer [27]. TFEB-dependent autophagy induces the progression of emphysema [28]. In this study, TLR4/TFEB signaling participated in M. pneumoniae-induced inflammatory response and autophagy of macrophages. Knockdown of TLR4 suppressed the expression of TFEB and autophagy, which was alleviated by the inhibitor of TEBF (CCl-779). Therefore, TLR4/TFEB may play an essential role in M. pneumoniae-induced inflammation and autophagy. Moreover, recent studies reveal that SARS-CoV-2 takes advantage of the lysosomal/endosomal system to infect cells and that a paucity of SARS-CoV-2 infections among patients is followed in their Gaucher clinics.
Figure 6: TFEB promoted *M. pneumoniae*-induced autophagy. (a) The expression of LC3 II detected by immunofluorescence. 3-MA suppressed the upregulation of LC3 puncta induced by *M. pneumoniae*. (b) Quantification of A. (c) The protein expression detected by western blot. Downregulation of TFEB reversed the increase of LC3 II induced by *M. pneumoniae*. (d) Quantification of C. (e) The expression of LC3 II detected by immunofluorescence. TFEB knockdown suppressed the upregulation of LC3 II induced by *M. pneumoniae*. **P < 0.01, ##P < 0.01."
References

[1] R. Dumke, S. Stolz, E. Jacobs, and T. Juretzek, “Molecular characterization of macrolide resistance of a Mycoplasma pneumoniae strain that developed during therapy of a patient with pneumonia,” International Journal of Infectious Diseases, vol. 29, pp. 197–199, 2014.

[2] E. Kassisse, H. García, L. Prada, I. Salazar, and J. Kassisse, “Prevalence of Mycoplasma pneumoniae infection in pediatric patients with acute asthma exacerbation,” Archivos Argentinos de Pediatría, vol. 116, no. 3, pp. 179–185, 2018.

[3] H. Lee, K. W. Yun, H. J. Lee, and E. H. Choi, “Antimicrobial therapy of macrolide-resistant Mycoplasma pneumoniae pneumonia in children,” Expert Rev Anti Infect Ther, vol. 16, pp. 23–34, 2018.

[4] Y. F. Wu, Z. Y. Li, L. L. Dong et al., “Inactivation of mTOR promotes autophagy-mediated epithelial injury in particulate matter-induced airway inflammation,” Autophagy, vol. 16, no. 3, pp. 435–450, 2020.

[5] S. N. Borchsenius, I. E. Vishnyakov, O. A. Chernova, V. M. Chernov, and N. A. Barlev, “Effects of mycoplasmas on the host cell signaling pathways,” Pathogens, vol. 9, no. 4, p. 308, 2020.

[6] M. Higuchi, A. Matsuo, M. Shingai et al., “Combinational recognition of bacterial lipoproteins and peptidoglycan by chicken Toll-like receptor 2 subfamily,” Developmental & Comparative Immunology, vol. 32, pp. 147–155, 2008.

[7] T. Shimizu, “Inflammation-inducing factors of Mycoplasma pneumoniae,” Frontiers in Microbiology, vol. 7, p. 414, 2016.

[8] R. S. T. Tan, B. Ho, B. P. Leung, and J. L. Ding, “TLR cross-talk confers specificity to innate immunity,” International Reviews of Immunology, vol. 33, no. 6, pp. 443–453, 2014.

[9] H. V. Le and J. Y. Kim, “Stable Toll-Like Receptor 10 knockdown in THP-1 cells reduces TLR-ligand-induced proinflammatory cytokine expression,” International Journal of Molecular Sciences, vol. 17, no. 6, p. 859, 2016.

[10] S. Shukla, T. T. Richardson, M. G. Drage, W. H. Boom, and C. V. Harding, “Mycobacterium tuberculosis lipoprotein and lipoglycan binding to Toll-Like Receptor 2 correlates with agonist activity and functional outcomes,” Infection and Immunity, vol. 86, no. 10, pp. e00450–18, 2018.

[11] M. Taghavi, E. Mortaz, A. Khosravi et al., “Zymosan attenuates melanoma growth progression, increases splenocyte proliferation and induces TLR-2/4 and TNF-alpha expression in mice,” Journal of Inflammation, vol. 15, no. 1, p. 5, 2018.

[12] D. Verzola, A. Bonanni, A. Sofia et al., “Toll-like receptor 4 signalling mediates inflammation in skeletal muscle of patients with chronic kidney disease,” Journal of Cachexia, Sarcopenia and Muscle, vol. 8, no. 1, pp. 131–144, 2017.

[13] T. Shimizu, Y. Kimura, Y. Kida et al., “Cytadherence of Mycoplasma pneumoniae induces inflammatory responses through autophagy and toll-like receptor 4,” Infection and Immunity, vol. 82, no. 7, pp. 3076–3086, 2014.

[14] Y. Wang, H. Li, Y. Shi et al., “miR-143-3p impacts on pulmonary inflammatory factors and cell apoptosis in mice with mycoplasmal pneumonia by regulating TLR4/iNOS/NF-kappaB pathway,” Bioscience Reports, vol. 40, no. 7, Article ID BSR20193419, 2020.

[15] L. Yu, M. Lu, W. Zhang, A. A. Alrafi, A. H. Hirad, and H. Zhang, “Ameliorative effect of Albizia chinensis synthesized ZnO-NPs on Mycoplasma pneumoniae infected pneumonia mice model,” Microbial Pathogenesis, vol. 141, Article ID 103960, 2020.

[16] J. W. Lee, H. Nam, L. E. Kim et al., “TLR4 (toll-like receptor 4) activation suppresses autophagy through inhibition of FOXO3 and impairs phagocytic capacity of microglia,” Autophagy, vol. 15, no. 5, pp. 753–770, 2019.

[17] W. Zhang, N. Zhuang, X. Liu et al., “The metabolic regulator Lamtor5 suppresses inflammatory signaling via regulating mTOR-mediated TLR4 degradation,” Cellular and Molecular Immunology, vol. 17, no. 10, pp. 1063–1076, 2020.

[18] Y. Ding, C. Chu, Y. Li et al., “High expression of HMGB1 in children with refractory Mycoplasma pneumoniae pneumonia,” BMC Infectious Diseases, vol. 18, p. 439, 2018.

[19] H. Luo, J. He, L. Qin et al., “Mycoplasma pneumoniae lipids license TLR-4 for activation of NLRP3 inflammasome and autophagy to evoke a proinflammatory response,” Clinical and Experimental Immunology, vol. 203, no. 1, pp. 66–79, 2020.

[20] A. Bahrami, V. Bianconi, M. Pirro, H. M. Orafaï, and A. Sahebkar, “The role of TFE3 in tumor cell autophagy: diagnostic and therapeutic opportunities,” Life Sciences, vol. 244, Article ID 117341, 2020.

[21] O. A. Brady, J. A. Martina, and R. Puertollano, “Emerging roles for TFE3 in the immune response and inflammation,” Autophagy, vol. 14, no. 2, pp. 181–189, 2018.

[22] N. Pastore, O. A. Brady, H. I. Diab et al., “TFEB and TFE3 cooperate in the regulation of the innate immune response in activated macrophages,” Autophagy, vol. 12, no. 8, pp. 1240–1258, 2016.

[23] G. R. Campbell and S. A. Spector, “Inhibition of human immunodeficiency virus type-1 through autophagy,” Current Opinion in Microbiology, vol. 16, no. 3, pp. 349–354, 2013.

[24] Y. Yue, N. R. Nabar, C. S. Shi et al., “SARS-Coronavirus Open Reading Frame 3a drives multimodal necrotic cell death,” Cell Death & Disease, vol. 9, p. 904, 2018.

[25] I. Vergne, F. Lafont, L. Expert, A. Esclatine, and M. Biard-Piechaczyk, “Autophagie, prot´ eines ATG et maladies infectieuses,” Medical Science, vol. 33, no. 3, pp. 312–318, 2017.

[26] C. Settembre, C. Di Malta, V. A. Polito et al., “TFEB links autophagy to lysosomal biogenesis,” Science, vol. 332, pp. 142914–142933, 2011.
[27] A. Giatromanolaki, M. Kouroupi, K. Balaska, and M. I. Koukourakis, "A novel lipofuscin-detecting marker of senescence relates with hypoxia, dysregulated autophagy and with poor prognosis in non-small-cell-lung cancer," *In Vivo*, vol. 34, pp. 3187–3193, 2020.

[28] M. Bodas, N. Patel, D. Silverberg, K. Walworth, and N. Vij, "Master autophagy regulator transcription factor EB regulates cigarette smoke-induced autophagy impairment and chronic obstructive pulmonary disease- emphysema pathogenesis," *Antioxidants and Redox Signaling*, vol. 27, pp. 150–167, 2017.

[29] P. Mistry, M. Balwani, D. Barbouth et al., "Gaucher disease and SARS-CoV-2 infection: emerging management challenges," *Molecular Genetics and Metabolism*, vol. 130, no. 3, pp. 164–169, 2020.

[30] L. Fierro, N. Nesheiwat, H. Naik, P. K. Mistry, M. Balwani, and M. Balwani, "Gaucher disease and SARS-CoV-2 infection: experience from 181 patients in New York," *Molecular Genetics and Metabolism*, vol. 132, no. 2, pp. 44–48, 2021.