Alveolar type II cells harbouring SARS-CoV-2 show senescence with a proinflammatory phenotype

Authors: Konstantinos Evangelou[1*], Dimitris Veroutis[1*], Periklis G. Foukas[2*], Koralia Paschalaki[3], Christos Kittas[1], Athanasios G. Tzioufas[4], Laurence de Leval[5], Demetris Vassilakos[1], Peter J Barnes[3#] and Vassilis G. Gorgoulis[1,6,7,8#]

[1]Molecular Carcinogenesis Group, Department of Histology and Embryology, Medical School, National and Kapodistrian University of Athens, Athens, Greece, [2]2nd Department of Pathology, Attikon University Hospital, Medical School, National and Kapodistrian University of Athens, Athens, Greece, [3]National Heart and Lung Institute, Imperial College London, London, UK, [4]Department of Pathophysiology, Medical School, National and Kapodistrian University of Athens, Athens, Greece, [5]Institute of Pathology, Lausanne University Hospital, Lausanne, Switzerland, [6]Faculty Institute for Cancer Sciences, Manchester Academic Health Sciences Centre, University of Manchester, Manchester, UK, [7]Biomedical Research Foundation, Academy of Athens, Athens, Greece, [8]Center for New Biotechnologies and Precision Medicine, Medical School, National and Kapodistrian University of Athens, Athens, Greece

[*]**equally contributed**

[#]**Corresponding Authors:**

Professor Peter J Barnes FRS, FMedSci

Airway Disease Section, National Heart & Lung Institute, Dovehouse St, London SW3 6LY, United Kingdom

phone: +44 20 7594 7959; fax: +44 20 7351 8126

email: p.j.barnes@imperial.ac.uk

Vassilis G Gorgoulis MD, PhD

Department Histology-Embryology, Medical School, National Kapodistrian University of Athens, 75 Mikras Asias Str, Goudi, Athens GR11527, Greece

phone: +302107462352

email: vgorg@med.uoa.gr

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Abstract

SARS-CoV-2 infection of the respiratory system can evolve to a multi-system disease. Excessive levels of proinflammatory cytokines, known as a 'cytokine storm' are associated with high mortality rates especially in the elderly and in patients with age-related morbidities. Senescent cells, characterized by secretion of such cytokines (Senescence Associated Secretory Phenotype - SASP), are known to occur in this context as well as upon a variety of stressogenic insults. Applying both: i) a novel "in house" antibody against the spike protein of SARS-CoV-2 and ii) a unique senescence detecting methodology, we identified for the first time in lung tissue from COVID-19 patients alveolar cells acquiring senescent features harboring also SARS-CoV-2. Moreover, using the same detection workflow we demonstrated the inflammatory properties of these cells. Our findings justify the application of senotherapeutics for the treatment or prevention of COVID-19 patients.

Introduction

Coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), primarily affects the respiratory system but can evolve to a multi-system disease with excessive levels of proinflammatory cytokines, described as a 'cytokine storm' (1). High mortality rates have been observed in the elderly and in patients with age-related morbidities (2). Understanding the underlying pathophysiological mechanisms is important in developing more effective therapies.

Cellular senescence is a fundamental mechanism characterised by generally irreversible cell-cycle arrest, altered metabolism, macromolecular damage and proinflammatory features termed Senescence Associated Secretory Phenotype (SASP) (3). The evidence linking viral infection to cellular senescence is sparse, although oncogenes, -which may trigger senescence- were initially discovered in viruses (4, 5). Viral infection has been associated with DNA damage and cell fusion, well-known inducers of senescence (4, 6), and elicits release of pro-inflammatory mediators that may promote senescence via paracrine mechanisms (5). Therefore, senescence may act as a cellular defense mechanism against viral infection and increased prevalence of senescence could occur in infected and surrounding cells. Given the significance of systemic inflammation in the outcome of COVID-19, we have studied whether SARS-CoV-2 may be associated with cellular senescence in infected lung cells and the SASP phenotype (5).

Methods

Patient samples: Formalin-Fixed-Paraffin-Embedded lung tissues from ten COVID-19 patients (mean±SD: 72.4±16yr; 7 males) confirmed by molecular testing, and ten age-matched non-COVID-19 lung tissues samples from previously published cohort (71.8 ± 15 yr, 5 males) were analyzed (7). All protocols were approved by the Commission cantonale d'éthique de la recherche, Lausanne (CERVD) Ref 2020-01257 and the Bio-Ethics Committee of Athens Medical School.

Immunocytochemistry: Immunohistochemistry was performed using: the anti-SARS-CoV-2 mAb (G2), which was produced and validated in house; anti-ACE-2 (Abcam); anti-Thyroid-Transcription-Factor(TTF)-1 (Dako); anti-CD68 (Dako); anti-$p16^{INK4A}$ (Santa Cruz); IL-1β (Abcam) and IL-6 (R&D systems), as described (8). The Novolink Polymer Detection System (Leica Biosystems) was used for development of the signal and hematoxylin for counterstaining. SenTraGor^{TM}(GL13) and double stainings were performed as published (8).

Quantification: Cells were considered positive with G2 staining irrespective of the staining intensity. A previously described semi-quantitative IHC evaluation was adopted (9). *Electron microscopy* was performed using a FEI Morgagni 268 transmission electron microscope equipped with Olympus Morada digital camera.

Results

We initially developed monoclonal antibodies against the spike protein for the detection of SARS-CoV-2, and the antibody with the highest affinity (G2) was used. The specificity of G2 mAb was validated in formalin fixed paraffin lung tissues from COVID-19 patients by: i) omitting it and ii) performing competition with the corresponding spike anti-peptide **(Figure 1A).** Absence of G2 immunoreactivity was observed in a large cohort of non-COVID-19 lung tissues (**Figure 1A,vii**) (7). SARS-CoV-2 was detected in alveolar type-II (AT2) pneumocytes (TTF-1 positive, CD-68 negative) of all COVID-19 patients, with values ranging from <5/4mm^2 to >50/4mm^2(data not shown). The SARS-CoV-2 infected AT2 cells were occasionally of large size with a brown moderate to strong, diffuse or granular cytoplasmic signal (**Figure 1Ai,iii,v**). Topologically, they either covered the alveolar walls and protruded into the airspaces from the edges of the alveolar septa or appeared within the alveolar spaces ısolated (denuded or syncytial) or in clusters (hyperplasia) (**Figure 1Ai,iii,v**). Electron microscopy analysis in representative cases confirmed the presence of the virus within AT2 cells (**Figure 1Bi,ii**). High magnification revealed virions in the proximity of the endoplasmic reticulum suggesting their assembly and budding, as well as virions residing in cytoplasmic vesicles indicating their transfer and release into the extracellular space (**Figure 1Biii,iv,v**).

Subsequently, we asked whether the G2-positive cells exerted features of cellular senescence. Applying the recently reported guideline workflow for senescence detection, we observed both in serial sections and through co-staining, a proportion of G2-positive AT2 cells (range 8 to 17%) displaying strong reactivity to SenTraGor, a marker of senescence (**Figure 2A,Bi,2C,D)** (3, 8). Senescent phenotype was further verified with co-

staining for $p16^{INK4A}$, a marker of stress-related senescence (**Figure 2Bii)**(3). Occasionally these cells tended to cluster (**Figure 2Ai-vi**) and in serial sections were found to co-express the ACE2 (**Figure 2Aiii,vi**), a finding supportive of SARS-CoV-2 infection mediated by ACE2 (1).

We next tested whether the senescent pneumocytes exert features of SASP. We found in serial sections and co-staining analysis (**Figure 2E-F**), expression of both IL-1β and IL-6, which were absent in non-COVID19 cases (**Figure 2G**). This observation provides the foremost verification for expression of SASP factors by senescent cells in an *ex-vivo* setting. As both cytokines are key components of the "cytokine storm", this implicates cellular senescence via SASP in the poor clinical outcome of COVID-19 patients who are elderly and suffering from age-related diseases.

Discussion

To the best of our knowledge, we provide the first evidence of cellular senescence and expression of SASP components in SARS-CoV-2 infected human lung cells. Moreover, we demonstrate that in the COVID-19 setting, senescence alters the properties and function of a respiratory cell compartment, rendering its constituents capable for producing cytokines that can readily be released into the systemic circulation. Whether senescence pre-exists at the time of infection, making cells more susceptible to infection with the virus, or whether senescence is triggered as an antiviral response, remain unanswered questions. Putatively, both scenarios could co-exist. Regarding the first scenario at the time of infection the burden of senescent cells is anticipated high in the elderly and in individuals with age-related diseases, rendering them vulnerable to develop a pro-inflammatory phenotype. Notably, in our COVID-19 cohort the percentages of senescent cells were higher in patients over 73 years-old vs younger patients (Mann-Whitney test: $p<0.05$). Regarding the second scenario, senescence might be an antiviral response against SARS-CoV-2 and infected senescent cells could induce paracrine senescence in nearby cells, thus increasing the senescence load. Irrespective of the origin, both scenarios are related to SASP secretion that seems at least in part a source or even a trigger of the pro-inflammatory cytokines, commonly observed in the blood of COVID-19 patients.

Overall, the reported findings justify the application and promising findings of recent studies using senotherapeutics (senolytics, SASP modulators/inhibitors) for treatment or prevention of COVID-19 patients (10).

Disclosure/Conflict of Interest

The authors wish to declare no conflict of interest.

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References

1. Hu B, Guo H, Zhou P, Shi ZL. Characteristics of SARS-CoV-2 and COVID-19. *Nature reviews Microbiology* 2020: 1-14.

2. Del Valle DM, Kim-Schulze S, Huang HH, Beckmann ND, Nirenberg S, Wang B, Lavin Y, Swartz TH, Madduri D, Stock A, Marron TU, Xie H, Patel M, Tuballes K, Van Oekelen O, Rahman A, Kovatch P, Aberg JA, Schadt E, Jagannath S, Mazumdar M, Charney AW, Firpo-Betancourt A, Mendu DR, Jhang J, Reich D, Sigel K, Cordon-Cardo C, Feldmann M, Parekh S, Merad M, Gnjatic S. An inflammatory cytokine signature predicts COVID-19 severity and survival. *Nature medicine* 2020; 26: 1636-1643.

3. Gorgoulis V, Adams PD, Alimonti A, Bennett DC, Bischof O, Bishop C, Campisi J, Collado M, Evangelou K, Ferbeyre G, Gil J, Hara E, Krizhanovsky V, Jurk D, Maier AB, Narita M, Niedernhofer L, Passos JF, Robbins PD, Schmitt CA, Sedivy J, Vougas K, von Zglinicki T, Zhou D, Serrano M, Demaria M. Cellular Senescence: Defining a Path Forward. *Cell* 2019; 179: 813-827.

4. Baz-Martínez M, Da Silva-Álvarez S, Rodríguez E, Guerra J, El Motiam A, Vidal A, García-Caballero T, González-Barcia M, Sánchez L, Muñoz-Fontela C, Collado M, Rivas C. Cell senescence is an antiviral defense mechanism. *Scientific reports* 2016; 6: 37007.

5. Kelley WJ, Zemans RL, Goldstein DR. Cellular Senescence: Friend or Foe to Respiratory Viral Infections? *The European respiratory journal* 2020.

6. Chuprin A, Gal H, Biron-Shental T, Biran A, Amiel A, Rozenblatt S, Krizhanovsky V. Cell fusion induced by ERVWE1 or measles virus causes cellular senescence. *Genes & development* 2013; 27: 2356-2366.

7. Gorgoulis VG, Vassiliou LV, Karakaidos P, Zacharatos P, Kotsinas A, Liloglou T, Venere M, Ditullio RA, Jr., Kastrinakis NG, Levy B, Kletsas D, Yoneta A, Herlyn M, Kittas C, Halazonetis TD. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature* 2005; 434: 907-913.

8. Evangelou K, Lougiakis N, Rizou SV, Kotsinas A, Kletsas D, Muñoz-Espín D, Kastrinakis NG, Pouli N, Marakos P, Townsend P, Serrano M, Bartek J, Gorgoulis VG. Robust, universal biomarker assay to detect senescent cells in biological specimens. *Aging cell* 2017; 16: 192-197.

9. Schaefer IM, Padera RF, Solomon IH, Kanjilal S, Hammer MM, Hornick JL, Sholl LM. In situ detection of SARS-CoV-2 in lungs and airways of patients with COVID-19. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 2020; 33: 2104-2114.

10. Kirkland JL and Tchkonia T. Senolytic drugs: from discovery to translation. *J Intern Med* 2020; 10.1111/joim.13141.

Figure Legends

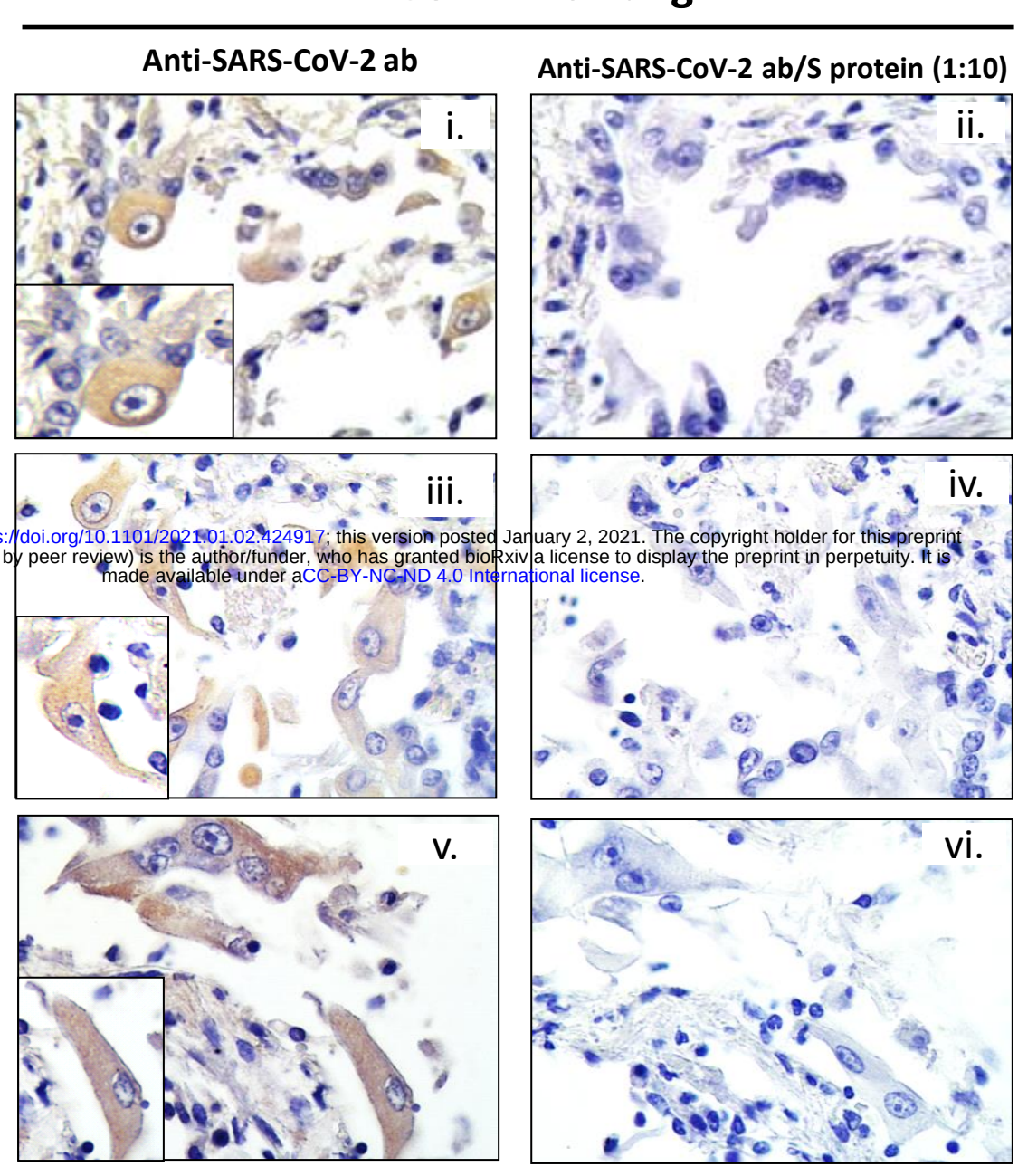
Figure 1: A. Immunohistochemistry (IHC) staining in COVID-19 lung tissue, showing cytoplasmic SARS-CoV-2 spike protein (brown color) with hematoxylin counterstain in alveolar type II (AT2) cells (DAB IHC) **(i,iii,v)**. Images from corresponding control experiments in COVID-19 lung tissue. Inclusion of the corresponding anti-peptide (S protein) in the primary antibody solution results in negative staining in serial sections **(ii,iv,vi)**. Original magnification: 400x, Insets 630x. **B.** Semi-thin section of the epoxy-embedded deparaffinized lung autopsy **(i)**. The Black box depicts a representative binucleated AT2 cell observed under electron microscope as shown in **ii.** Toluidine blue O staining. Original magnification: 200x**.** Electron micrograph of a binucleated enlarged pneumocyte showing round to oval nuclei and vacuolated appearance of the cytoplasm (**ii**). A drawing of the cytoplasmic and nuclear area of the cell in figure B, focusing to the steps of virions assembly and transferring to vesicles as shown at higher magnification **(iii)**. Higher magnification of the cytoplasmic area demarcated by the box in **ii**, showing virions (red arrows) in the proximity of RER, as well as in vacuoles (**iv,v,vi**). Cubic membrane structures arranged in an ordered fashion were rarely observed in the cytoplasm of infected cells (*red asterisk). Scale bars: Bii: 1µm; Biv-vi: 200nm**.** *Abbreviations:* N: nucleus; n: nucleolus, RER: rough endoplasmic reticulum.

Figure 2: A. Representative images of G2 **(i)**, SenTraGor **(ii)** and ACE-2 **(iii)** staining in serial sections of COVID-19 lung tissue. **B.** Representative results from double-staining experiments showing cytoplasmic localization of viral spike protein in cells that are

concurrently positive with SenTraGor (senescent) **(i),** and nuclear $p16^{INK4A}$ expression in cells that are concurrently positive with SenTraGor **(ii)**. B(i) left inset depicts an AT2 cell solely exhibiting cytoplasmic immunopositivity for the viral Spike protein. **C.** Representative images of G2 **(i)**, SenTraGor **(ii)** and ACE-2 **(iii)** staining in serial sections of non COVID-19 "normal" lung parenchyma in the vicinity of a tumor (n=10, aged matched with cases presented in Fig 2A). Range of SenTraGor labeling indices: 1-2%. Morphologically, senescent AT2 cells in non COVID-19 tissue exhibit a decreased size in relation to those in COVID-19 cases**. D.** Graph depicting differences in SenTraGor staining between non-COVID19 and COVID19 cases, ***p<0.001 (Mann-Whitney U test). **E.** Representative images of Interleukin 1β (IL-1β) **(i)**, SenTragor **(ii)** and Interleukin-6 (IL-6) **(iii)** in serial sections of COVID-19 lung tissue. **F.** Representative results from double-staining experiments showing cytoplasmic localization of IL-1β in cells that are concurrently positive with SenTraGor **(i),** and IL-6 expression in cells that are concurrently positive with SenTraGor **(ii)**. **G.** Representative images of IL-1β **(i)**, SenTraGor **(ii)** and IL-6 **(iii)** in serial sections of non-COVID-19 lung tissue (see also C). Original magnification: 400x, Insets 630x; Hematoxylin and nuclear fast red counterstain (Bi and F); DAB IHC – brown color; In co-staining SenTraGor was visualized with the BCIP/NBT chromogenic hybrid Histo-IHC reaction (dark blue perinuclear and cytoplasmic colour).

FIGURE 1

A.



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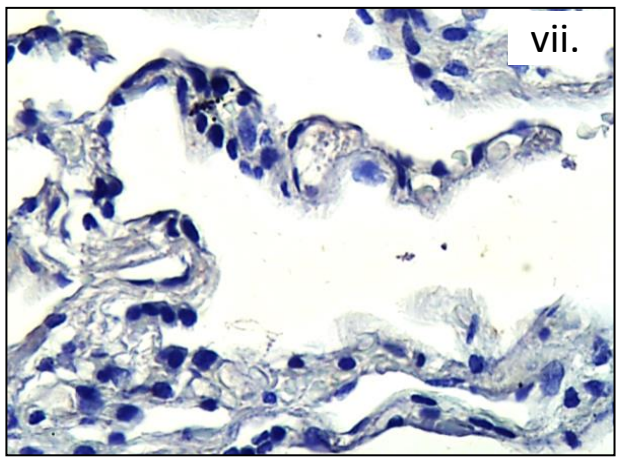

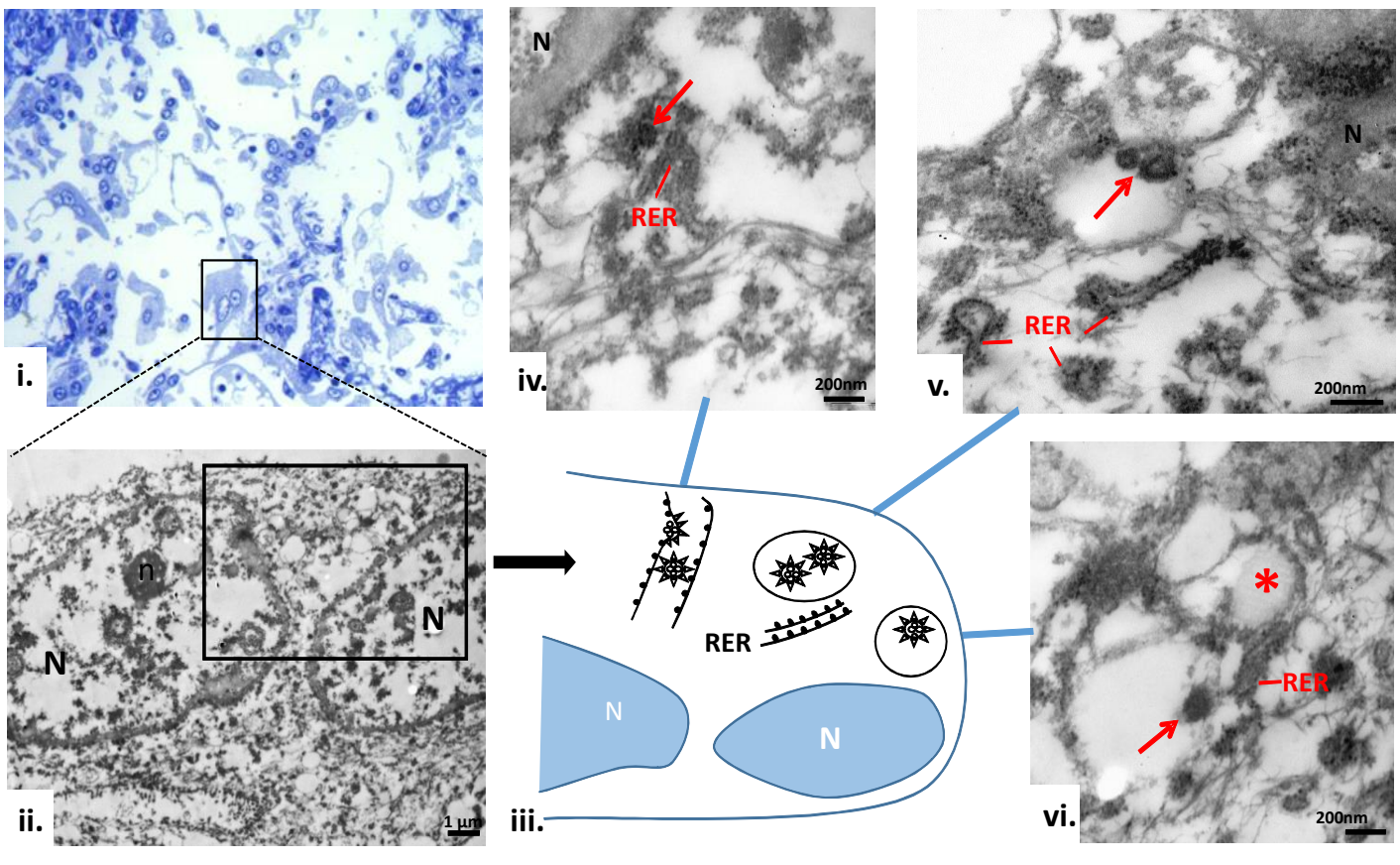

FIGURE 2.