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# Histopathological examination of lung from infant with lethal COVID-19 with special attention on pneumocytes type II and the immune infiltrate: a case study

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## Abstract

**Background** COVID-19 is a complex disease caused by SARS-CoV-2. The molecular and cellular mechanisms of the disease are unclear and their study is one of the greatest challenges for the modern science. Since the lung is the biggest target for SARS-CoV-2, the studies on cellular and molecular changes in this organ are essential to establish the pathogenesis of the disease. To date there is increasing number of reports on the lung pathology of fatal COVID-19 and the results are mainly obtained by autopsies of elderly patients, since this age group shows highest mortality. Little is known about the progression of the disease in children and especially newborn and infants and, to our knowledge, there are no reports on the lung features of fatal COVID-19 in this age group.

**Methods** In the present case study, we have investigated the lung morphological features in 11-months old infant who has died as a result of complications from COVID-19. Immunohistochemistry for immune cell markers and transmission electron microscopy for alveolocytes type II (ATII) are made.

**Results** Immediate cause of the death was acute respiratory failure resulting from bilateral interstitial pneumonia and subsequent acute cardiovascular failure. The histopathology shows lung edema, hyaline membranes, airway mucus plugging and interstitial inflammation. On cellular level we have observed a substantial increase in the number of ATII cells. ATII cells were marked with cytokeratin 19, TTF1 and napsin A. Transmission electron microscopy reveals ongoing apoptosis in these cells with a typical chromatin clustering and condensation towards the inner nuclear membrane. Immunohistochemistry shows significant increase of CD68+ macrophages in the alveoli, increase of IL-6 in immune and stromal cells, moderate elevation of FOXP3+ and IL-17+ cells and expression of CD4+ and CD8+ cells in alveolar walls. Immune cell interactions are discussed in the sense of ongoing cytokine storm.

**Conclusions** Our findings highlight the complexity of COVID-19 lung affection, involving ATII cell hyperplasia, interstitial mononuclear cell infiltration and macrophages increase. The findings provide an additional knowledge on the pathophysiology of COVID-19 in the lung and can serve as a basis for investigation of molecular mechanisms of this disease.

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**Keywords** Alveolar type II cells, SARS-CoV-2, Infant, Immune cells, Pulmonary disease, IL-6, Cytokine storm

## Introduction

COVID-19 (Coronavirus disease 2019) is a complex disease caused by SARS-CoV-2 (Severe Acute Respiratory Syndrome-Coronavirus 2 [1–3].

The pediatric group represents about 1% of all hospitalized cases and deaths [4]. The contemporary data about management, comorbidities, prevention and therapy are reported [5].

Although rare in children, COVID-19 pneumonia varies in severity from asymptomatic or paucisymptomatic disease to a factor significantly increasing the length of hospitalization [6]. The first report of 10 children with COVID-19 infection shows epidemiological and clinical features and 30.8% have had lung damage [7]. The main symptoms in 171 children with COVID-19 are announced as cough (48.5%), fever (41.5%), diarrhea (8.8%), and vomiting (64%) [8]. Another study shows that 11 out of 41 children hospitalized with COVID-19 present the picture interstitial pneumonia [9].

The molecular and cellular mechanisms of the disease are unclear and their study is one of the greatest challenges for the modern science. Since the lung is the biggest target for SARS-CoV-2, the studies on cellular and molecular changes in this organ are essential to establish the pathogenesis of the disease. Results obtained from autopsies have been crucial for the identification of the pathological mechanisms of lethal COVID-19 and have provided important information on the pathogenesis of this disease and the appropriate treatment approaches. Based on results obtained by autopsies, the pathomorphological features of fatal COVID-19 have been described by many groups worldwide [10–15] and its morphological progression into the lung tissue has been established. Pulmonary COVID-19 can be divided into 4 main morphological stages: (1) an early stage (day 0–1) with edema, epithelial damage, and capillaritis/endothelitis, (2) the stage of exudative diffuse alveolar damage (days 1–7), (3) the organizing stage (1 to several weeks) and (4) the fibrotic stage of diffuse alveolar damage (weeks to months) [13]. Histopathology of the lungs showed that diffuse alveolar damage is consistent with early acute respiratory distress syndrome, hyaline membranes formation, and activation of pneumocytes type II, progressing to their hyperplasia [15, 16].

In this regard, the ATII cells, also called alveolocytes type II or pneumocytes type II are of particular importance to study the cellular and molecular mechanisms of COVID-19 infection. This is supported by the following facts: (1) ATII cells are a target for SARS-CoV-2 because they express on their surface an angiotensin-converting enzyme 2 (ACE2) receptor for the spike protein (S) of the virus [17, 18]; (2) ATII cells secrete surfactant which stabilizes the alveoli and prevents their collapse on exhalation. Dysfunction or deficiency of this surfactant as a result of destruction of ATII cells by SARS-CoV-2 would lead to alveolar collapse, and a number of pathophysiological, morphological and clinical manifestations [19]; (3) Disruption of ATII cells would facilitate the infiltration of monocytes into the lung that may lead to a rapid and intense cytokine storms. The latter can cause tissue damage and affect the adaptive immune response expressed in cellular infiltration of lymphocytes and monocytes in lungs, thus leading to a multi-organ failure and death [20]; (4) Since ATII cells are responsible for regeneration of alveolar type I (ATI) cells, the apoptosis of ATII cells will lead to reduction in number of ATI cells, causing denuding of the alveolar wall, and leading to the development of fibrosis and parenchymal remodeling [21].

Besides their implication in the COVID-19 pathogenesis, the ATII cells have important physiologic and pathologic activities in other lung diseases such as Chronic obstructive pulmonary disease, lung adenocarcinoma etc [22]. At present, various immunohistochemical markers have been introduced to study the presence and localization of the ATII cells. Amongst them, thyroid transcription factor 1 (TTF-1) [23–25],, cytokeratin 19 (CK-19) [26] and napsin A [27] have proved to be the most reliable.

Immune cell infiltration is generally considered as being causally linked to the progression of inflammation and the outcome of the disease. The distribution of T and B lymphocytes and macrophages in lungs have been widely examined [10, 20, 28, 29]. Amongst the immune cells, the lung macrophages have received a considerable attention as an important innate immune cells involved in both the normal physiological functions and acute and chronic lung infections diseases [20, 30]. The presence of many macrophages in alveoli and alveolar walls is a characteristic feature of SARS-CoV-2 infection [31, 32]. It has been shown that the alveolar monocytes/macrophages also express angiotensin-converting enzyme 2 (ACE2) receptor for the S protein of COVID-19 virus, hence they can engulf and carry the virus [28, 30]. Sars-Cov-2 viruses replicate into these macrophages a phenomenon, called "the macrophage paradox" [30]. Macrophages are also crucial in triggering the "cytokine storm", since they secrete IL-1β, IL-6, IL-10, and TNFα cytokines and chemokines such as CCL2, CCL3 and CCL4 [31, 33].

There is a relationship between the ATII cells and lung alveolar macrophages since the latter are essential for the catabolism of surfactant generated by ATII cells [34].

The post-mortem examinations are still most reliable approach to study the circumstances of COVID-19 infection. The autopsies are mainly done on elderly patients, since the incidence of severe COVID-19 increased with the age and older patients show highest mortality. Children seem to be less susceptible to COVID-19 infection, hence the studies on the impact of this disease on young patients are scarce, while the potential harm of COVID-19 infection in neonates and infants remains largely unknown [35–37]. This is also due to the lack of autopsy studies on newborn or infants, since the mortality amongst this age group as a result of COVID-19 infection is practically reduced to zero.

Here, we report a single case about 10-months old infant died of COVID-19. We analyzed his lung tissue and determined the potential correlation between lung pathological changes and cellular aspects of the damage.

Our hypothesis is to study the pathogenesis of local lung immune responses to infection that is fundamental to control viral infection.

## Materials and methods

### Patient

A 10-month-old male child has been hospitalized in the Clinic of Infectious Diseases at University hospital "Prof. Dr. Stoyan Kirkovich", Stara Zagora, Bulgaria, on 14.10.2022 treated for 18 days and died on 01.11.2022.

He has been admitted to an Infectious Diseases Clinic with a history of diarrheal stools of more than 10 per day for the past 3 days. The rapid test for COVID-19 has been positive. At the time of examination the temperature is within normal values. The tongue is dry, the skin is pale, with marked perioral cyanosis. There is weakly expressed subcutaneous fat tissue, hypotrophy and third-degree protein-energy deficiency. Tachypnea and dyspnea with intercostal and epigastric retraction are observed. Auscultation reveals single small moist rales at the bases and decreased vesicular breathing. The respiratory rate is 50/min, the heart rate – 75/min. The abdomen is soft, with physiological peristalsis, without hepatosplenomegaly. There are no symptoms of meningeal-radicular irritation.

X-rays of the lungs are taken twice. Bilaterally expanded lung parenchyma with ground glass appearance is seen. There is presence of inhomogeneous striped-spotted inflammatory-infiltrative changes in the parenchyma and small areas of bilateral parenchymal consolidation subpleurally. The described changes are due to interstitial COVID-19 pneumonia (Fig. 1A). Following X-ray examination shows ground glass appearance, and larger areas of parenchymal consolidation corresponding to inhomogeneous striped-spotted inflammatory-infiltrative changes in the parenchyma (Fig. 1B).

Due to the anamnestic data on diarrheal syndrome, microbiological and serological examinations of the feces are performed - Salmonella, Shigella, *E.coli*, Rotavirus and Candida are not detected. The nasopharyngeal secretion is examined three times during the stay for COVID-19 by the PCR method– with positive results. There is difficult-to-control acidosis, with progressive leukocytosis with lymphopenia and worsening of the anemic syndrome, despite replacement therapy with biologics. Therapy includes also intravenous glucose-saline solutions, antibiotics - ceftriaxone and amikacin, methylprednisolone, diuretics, antipyretics, vitamins and probiotics. On the 16th day, a sharp deterioration in the patient's condition is recorded with manifestations of severe respiratory and cardiovascular failure with cyanosis, tachypnea and tachycardia. This necessitates his relocation to the intensive care unit and the inclusion of artificial pulmonary ventilation. Despite the active therapeutic and resuscitation measures, the child's condition progressively worsens and on the 18th day of the stay, it ends fatally.

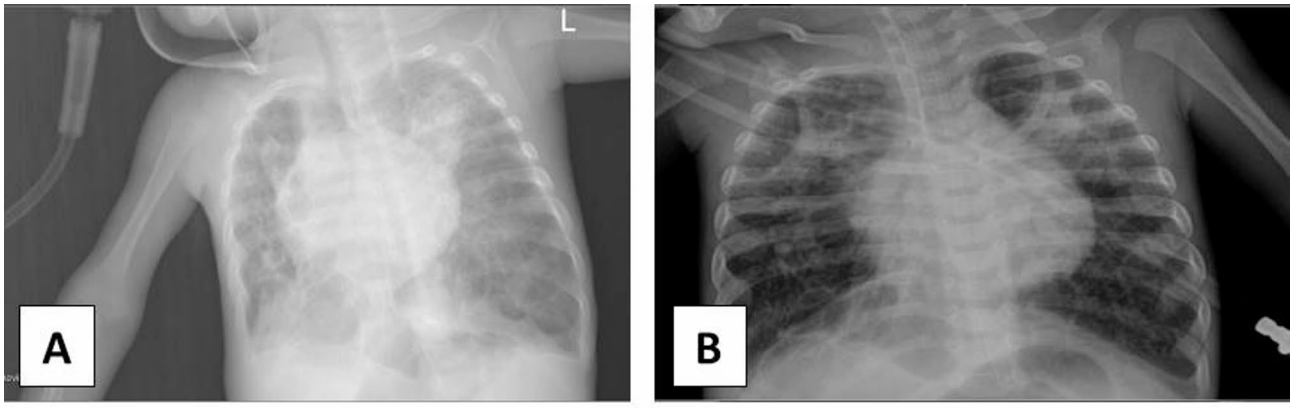


**Fig. 1** X-rays of the lungs were taken twice. On the 14th day of the stay, the presence of inhomogeneous striped-spotted inflammatory-infiltrative changes in the parenchyma, as well as increased transparency in the left basal region, was detected. (Fig. 1A). In the control study, the described changes are preserved. (Fig. 1B)

### Autopsy and specimen collection

The autopsy procedure is performed and pieces from each organ for analysis are collected. Lungs appear congested and edematous. About 18 pieces measuring 1 × 1.5 × 1 cm are collected from the lungs, fixed in 10% of neutral formaldehyde, processed and paraffin-embedded for light microscopy examinations. Pieces from lung measuring 0.2 × 0.3 × 0.2 cm are fixed in 0.2% glutaraldehyde, processed and finally embedded in epoxy resin for electron microscopy.

### Histological and histochemical examination

Histological sections, from 10% of neutral formaldehyde-fixed and paraffin-embeded lung tissue are stained with hematoxylin and eosin, and examined under light microscope (LEICA MC120 HD). Periodic acid Schiff (PAS) and Masson trichrome staining are carried out for detection of mucus and collagen fibers in the lungs.

### Immunohistochemistry

Method previously described by us [38] has been used. Briefly, tissue samples cut to 4 μm thickness are processed for light microscopy, dewaxed in xylenes at 56° C for 1 h, and rehydrated in alcohol and deionized water. The avidin-biotin-peroxidase complex technique has been used and the detection has been done on EnVision®TM FLEX + System, HRP K8002 (Agilent Technologies, Santa Clara, CA, USA). The reaction is visualized by a mixture of 3,3'– diaminobenzidine (DAB) (Sigma, St. Louis MO, USA). The sections are counterstained by Mayer's hematoxylin. Negative controls are elaborated using PBS instead of primary antibodies.

The following primary antibodies are used: macrophage marker CD68 (monoclonal mouse anti-human CD68, clone PGM1; IS613); T-lymphocyte marker CD3 (monoclonal rabbit anti-human CD3, clone SP7; IR503, ready-to-use); CD4 (monoclonal mouse anti-human CD4, clone 4B12; ready-to-use); CD8 (monoclonal mouse anti-human CD8, clone C8/144B; IR623, ready-to-use) all obtained from (Agilent Technologies, Santa Clara, CA, USA). Monoclonal mouse anti-human IL-17 sc-374,218, Lot N D2914, 1:100; monoclonal mouse anti-human IL-Foxp3 sc-53,876, Lot N H0618 1:50; monoclonal mouse anti-human STAT3 sc-8019, Lot N F1516, 1:100, all obtained from (Santa Cruz).

B lymphocyte marker used is CD20 (monoclonal mouse anti-human CD20cy, clone L26; IR624, ready-to-use) all from Dako and mouse anti-human IL-6 clone 10C12, 6,017,010 (Leica, Germany).

The markers for the ATII cells are as follows: mouse anti-human cytokeratin 19 clone RCK108, IR615; mouse anti human TTF-1, clone 8G763/1, IR056 (both from Dako) and mouse anti-human napsin A clone Cocktail RER A00131-0007 (LOT 60601) (Scy Tek, USA). Detection system used is EnVision®TM FLEX + System, HRP K8002 (Agilent Technologies, Santa Clara, CA, USA).

### Electron microscopy

Lung samples are fixed in 0.2% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.3, washed in 0.1 M PB, post-fixed in 1% $OsO_4$, and dehydrated in ascending alcohol series. The embedding, is done by a subsequent inclusion in propylene oxide, propylene oxide and durcopan and finally in durcopan in thermostat at 56°for 30 min. The blocks are sectioned using glass knives in a Leica UC 7 ultramicrotome (Leica Microsystems, Germany). Sections are collected on 200 mesh copper grids (Agar Scientific). They are subsequently stained for 10 min each with uranyl acetate and lead citrate and viewed in a Jeol 1400 TEM (JEOL Ltd., Japan). The electron microscopy is done at laboratory "Microscopy and Microanalysis Unit", University of Kwa-Zulu Natal, Durban, South Africa.

## Results

### Pathological observations after lung biopsy

Histological examination (H&E staining) reveals interstitial pneumonia consisting of mononuclear inflammatory cells in the alveolar septal walls, capillary stasis and discrete fibrosis, shown by trichrome Masson staining. The alveolar damage is evident by the presence of hyaline membranes. The alveolar edema fluid is noted focally. In the bronchioles sloughed respiratory epithelium and mucus plugs are present (mucous bronchiolitis) **(**Fig. 2A, B, C, D).In the alveoli pneumocytes type II (ATII cells) hyperplasia is observed. The cells are rounded with eosinophilic cytoplasm and large rounded basophilic nuclei. Some ATII cells have just completed mitosis.

*TTF-1* (Fig. 3). Most of the round epithelial cells overlaying the alveoli give a positive nuclear reaction with TTF-1 (Fig. 3A). Higher magnification shows that the TTF-1 marker is located in the nuclei of ATII cells (Fig. 3B). The ATII cells and their nuclei are within normal sizes. It should be noted that there is a substantial increase in number of the ATII cells covering the alveolar surface. Distinct white patches are observed within the nuclei of these cells (Fig. 3B).

*CK-19***(**Fig. 4**)** CK19 staining show a uniform strong positive reaction on the alveolar epithelium (Fig. 4A). Both ATI and ATII cells react equally with anti-CK19 antibody, however the ATII cells prevail over the ATI cells (Fig. 4B). Detached positively stained ATI and ATII epithelial cells, are observed in the alveolar space, suggestive of epithelial injury (Fig. 4A). In some instances, the alveolar surface is denuded, due to detachment of the ATI and ATII cells as result of severe epithelial damage.

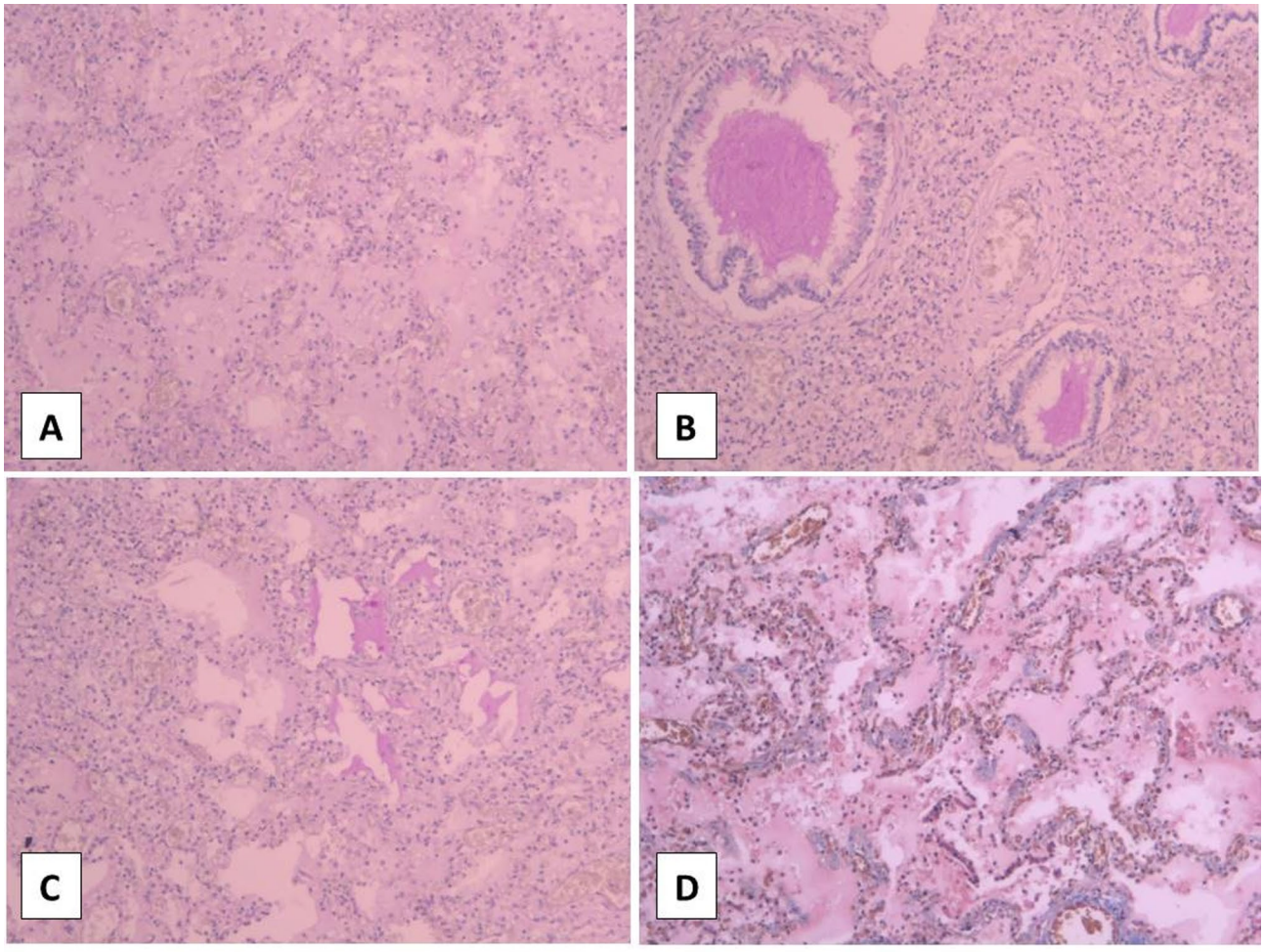


**Fig. 2** General pathological observations after lung biopsy and subsequent PAS (**A**–**C**) and Masson trichrome staining (**D**), x400. alveolar edema; (**B**) mucus plugs in the bronchioles; (**C**) -hyaline membranes and (**D**) fibrosis. Asterisk and arrows point to the corresponding damages

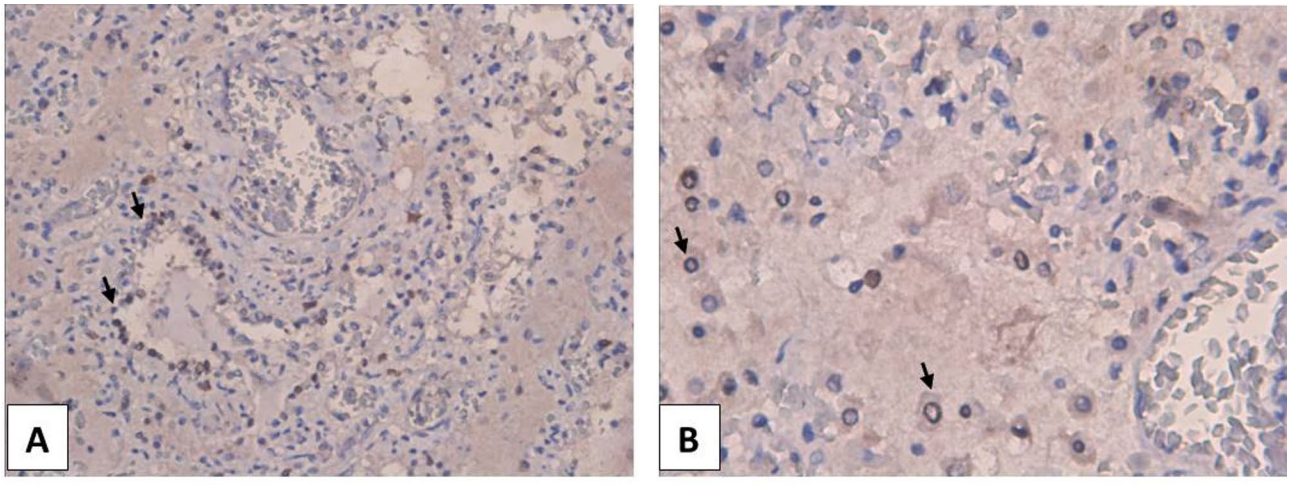


**Fig. 3** TTF-1 staining of lung parenchyma from lethal COVID-19 case. **A**, The alveoli are filled with edema fluid. A distinct layer of TTF-1 labeled cells is visible. **B**, Distinct, light patches are noted in the nuclei of AT-II cells (arrows). TTF-1; Thyroid transcription factor 1. A– x200; B– x400

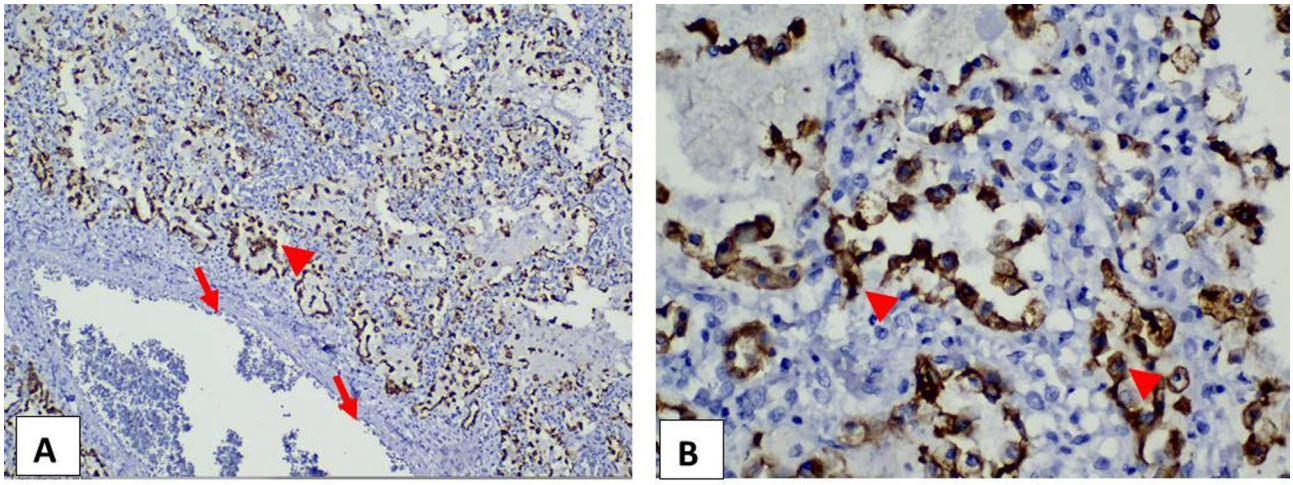


**Fig. 4** CK19 staining of lung parenchyma from lethal COVID-19 case. **A**. CK19 label is specifically confined to the alveolar epithelium, while the endothelium of the blood vessels is free of label (arrow). **B**. Both ATI and ATII cells show a uniform, strong, positive reaction to CK19 label. The prevailing number of ATII cells over the ATI is obvious. A– x 100; B,– x400. Arrows point to the ATI cells, while arrowheads to the ATII cells

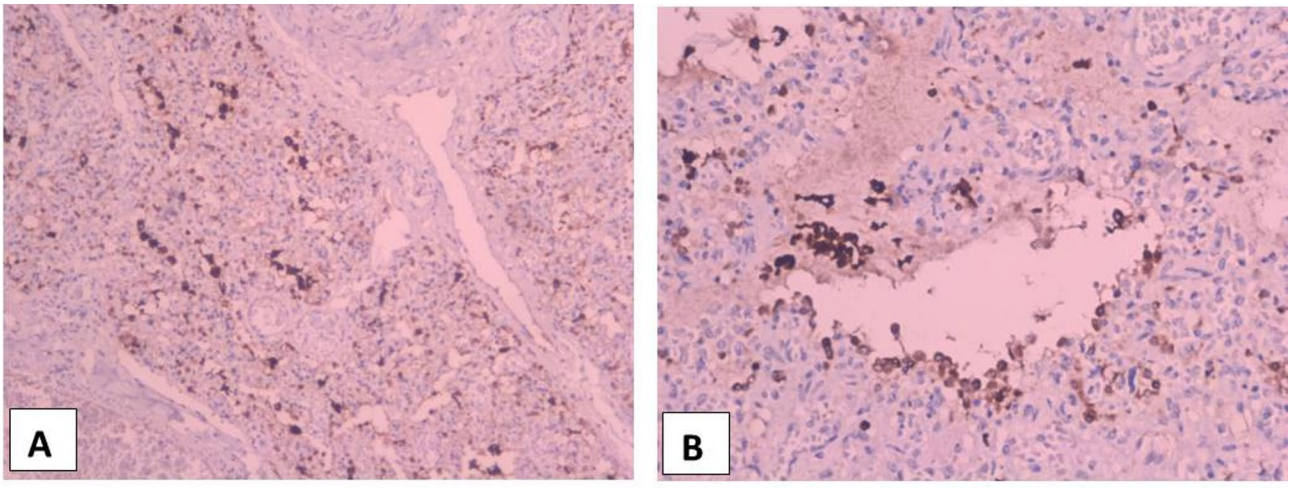


**Fig. 5** Immunohistochemical staining with napsin A revealed a predominant localization of this antibody in the cytoplasm of ATII cells. **A**– x100; **B**– x200

*Napsin A* (Fig. 5A, B) Immunohistochemical staining with napsin A reveals a predominant localization of this antibody in the cytoplasm of ATII cells.

#### TEM observations

TEM micrographs of ATII cells in lung tissue obtained from lethal COVID-19 case show highly condensed heterochromatin clusters, indicating an onset of apoptosis. Majority of the cells are still functional, as indicated by the presence of intact lamellar bodies as well as an extensive exocytosis (most probably of surfactant) into the alveolar cavity (Fig. 6A). In other cells the apoptosis is more advanced as seen by the highly irregular shape of the nucleus and heterochromatin withdrawal towards the periphery of the nucleus. Nevertheless, the cell membrane is still intact and single microvilli typical for ATII cells are observed (Fig. 6B). Despite the distinct changes in the nucleus, the laminar bodies appear well preserved, although an extensive vacuolization is observed in the cytoplasm (Fig. 6C, D). Other cell organelles like mitochondria and Golgi apparatus also appear intact (Fig. 6D).

#### Lung inflammation markers

The alveoli show enlarged alveolar septal walls with mononuclear cell infiltration (Fig. 7A). $CD4^+$ T cells, $CD8^+$ T cells, $STAT3^+$ cells, and $CD68^+$ macrophages are seen there. T lymphocytes and B lymphocytes are reduced and $CD68^+$ macrophages are increased in alveoli and in the alveolar wall. The lymphoid aggregates show intense $CD3^+$, $CD4^+$, and $CD8^+$ (Fig. 7B) and dispersed $CD68^+$ macrophages. $IL\text{-}6^+$ immune cells (macrophages

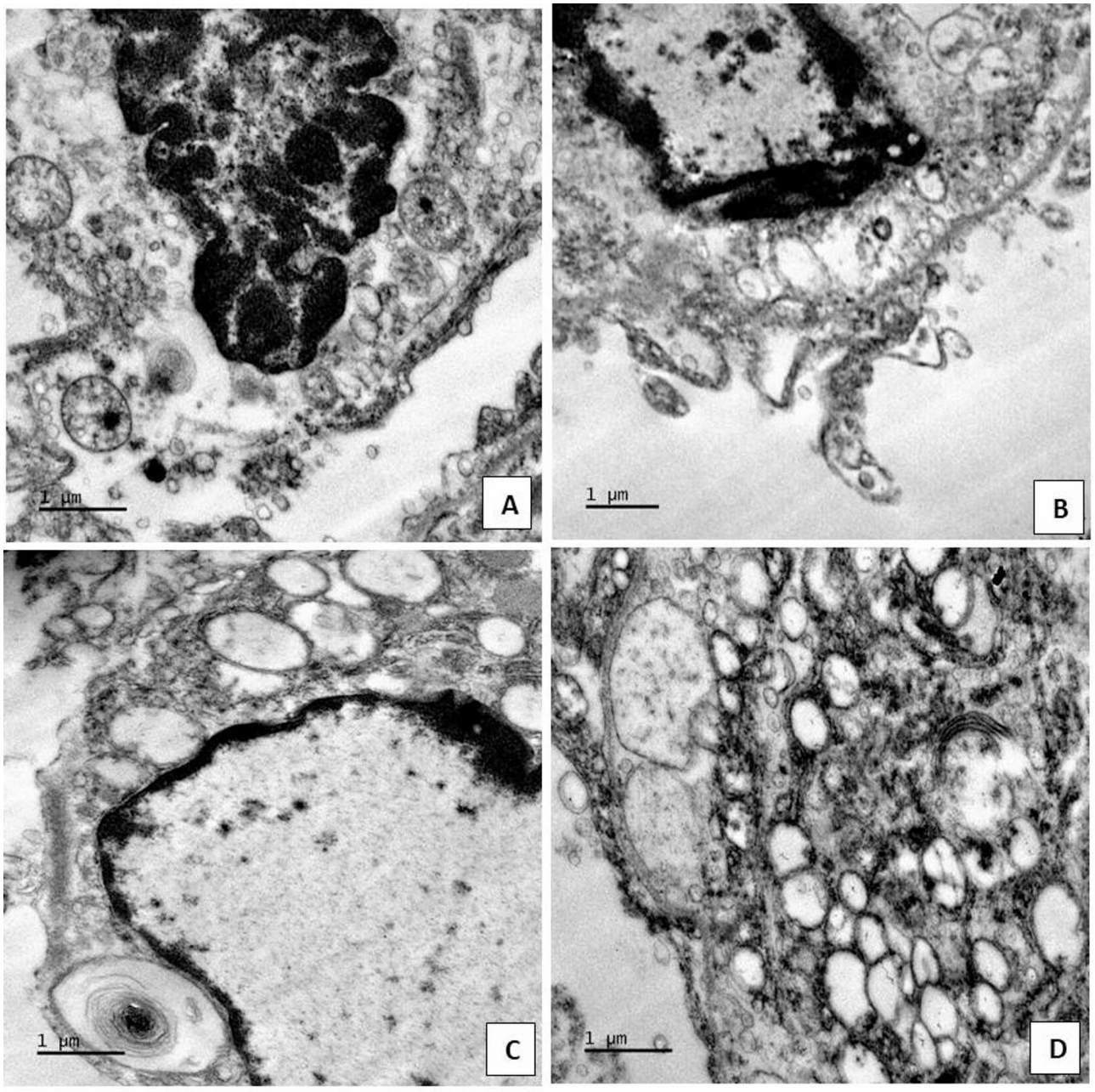


**Fig. 6** TEM micrographs of AT II cells in lung tissue obtained from lethal COVID-19 case. **(A)** Cell with highly condensed heterochromatin clusters, indicating an onset of apoptosis. The mitochondria and lamellar bodies appear intact. Note the exocytosis (thick arrow) from the AT II cell into the alveolar cavity. **(B)** Cell in advanced apoptosis as indicated by the highly irregular shape of the nucleus and heterochromatin withdrawal towards the periphery of the nucleus. The arrowhead points to the single microvillus on the surface of the cell. **(C)** Despite the distinct changes in the nucleus, the lamellar bodies appear intact. **(D)** There are no visible changes in the ultrastructure of the mitochondria and Golgi apparatus, but extensive accumulation of vacuoles was evident. A– alveolar lumen; H- heterohromatin; GA - Golgi apparatus; LB– lamellar body; M– mitochondrion; Nu– nucleus; V– vacuole

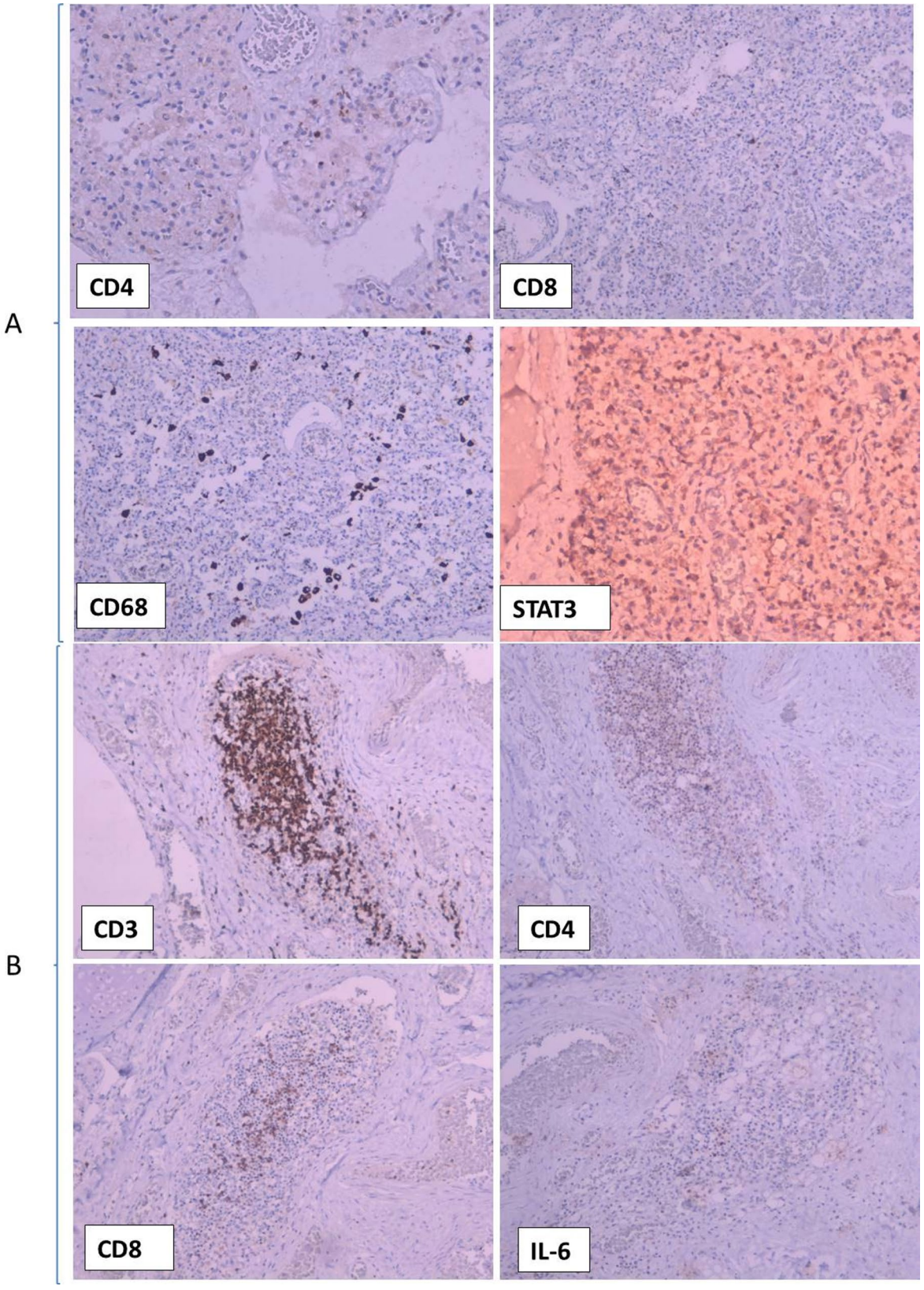


**Fig. 7** **(A)** Immune cells in the alveolar walls: $CD4^+$, $CD8^+$, $CD68^+$, $STAT3^+$; **(B)** Immune cells in the peribronchial TLS: $CD3^+$, $CD4^+$, $CD8^+$, $IL\text{-}6^+$. A– x200; B– x100

and lymphocytes) (Fig. 7B) are found in lymphoid aggregates. FoxP3 + Tregs and IL-17 + T helpers (Th17) cells are numerous in the alveoli (Fig. 8A, B).

## Discussion

Our study presents a systemic evaluation of lung alveolar type II cells, immune cells such as T and B lymphocytes, macrophages and IL-6$^+$ cells using light microscopy, immunohistochemistry and transmission electron microscopy. There are a number of reasons for the severe course and fatal outcome in the described clinical case. The patient's respiratory failure, aggravated by pneumonia, led to respiratory acidosis, unresponsive to medical treatment, oxygen therapy, and subsequently - to artificial lung ventilation. It has been established that in children under two years of age, the probability of developing a severe COVID-19 infection is significantly greater [39, 40]. In most cases of children with COVID-19 and diarrheal syndrome, the disease is mild, often self-limiting [41, 42]. But there are also more severe cases, associated with higher levels of inflammatory markers, suggesting a stronger immune response of the body [43]. In our case, there is a severe diarrheal syndrome, worsening dehydration and metabolic acidosis, despite active therapy - intravenous rehydration and symptomatic antidiarrheal agents. This, together with the underlying anemic syndrome and the existing protein-energy deficiency, determine a more protracted course and the likelihood of a poor outcome [44, 45]. Concerning X-ray examination it is the first preferred imaging method in pediatric patients with COVID-19 [46]. Our finding of presence of bilateral increase in density and ground-glass changes is characteristic in 60% of children with coronavirus infection [47].

On a cellular level we observe a substantial increase in the number of alveolar type II (ATII) cells in the vicinity of the alveoli, suggestive of hyperplasia of these cells [48]. In some instances, the alveolar epithelium is denuded and numerous detached ATI and ATII, as well as ATI (alveolar type I) cells are observed in the alveolar cavities, which is a sign of progressive epithelial injury. Light microscopy reveals distinct transparent areas within the nuclei of still intact ATII cells after histochemical labelling with TTF-1. Transmission electron microscopy (TEM) reveals ongoing apoptosis in these cells with a typical chromatin clustering and condensation towards the inner nuclear membrane [49, 50]. Surprisingly, the surfactant producing lamellar bodies within the ATII cells are intact, suggesting that the immediate cause of the infant's death is not the lack of surfactant [51].

**Pathologic findings** in pulmonary tissues of COVID-19 have been described [10, 52]. Previously, the pathologic features of SARS-CoV-2 have been shown [14, 50, 53] and a similar histopathology of the COVID-19 pulmonary disease and SARS-CoV-2 severe acute respiratory syndrome from 2003 have been found [52]. In our case, pulmonary pathology consisted of interstitial mononuclear cell infiltration, capillary stasis, sloughing of epithelial cells, hyperplasia of ATII cells and macrophage infiltration in the alveoli. Scarce hyaline membranes in the alveoli are also formed. The latter, together with the lymphocyte infiltrated alveolar walls lead to gas exchange obstruction. Hyaline membranes have been considered to be uncommon in COVID-19 lungs of elderly patients [52]. The presence of mucus plugs in respiratory tract in our case is in accordance with the findings of other authors [52, 54], who describe it as a unique feature of COVID-19 histopathology in lungs. Unlike other authors [11], however, we do not see thromboembolic events in pulmonary arterioles.

Our histological and immunohistochemical fundings reveals mononuclear inflammation within bronchioli and

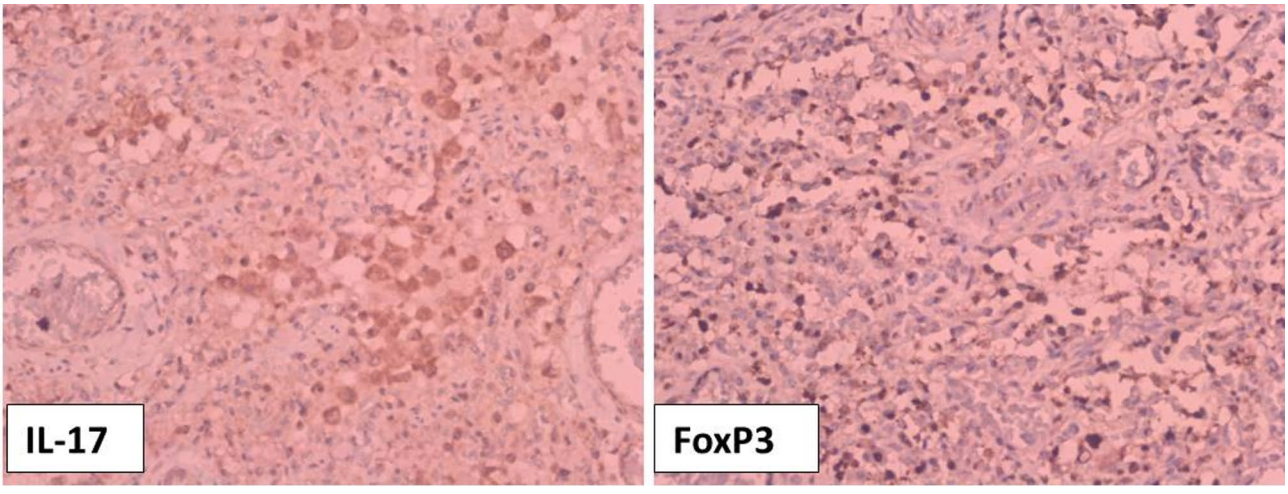


**Fig. 8** Increased numbers of Th17 cells (IL-17) and T-reg cells (FoxP3) in alveoli. X100

bronchi with prominent mucosal edema. A peribronchial lymphoid aggregation is noted, known as tertiary lymphoid structure, consisting mainly of $CD3^+$ T lymphocytes and sparsely of $CD4^+$and $CD8^+$ T lymphocytes and $CD20^+$ B lymphocytes. $CD68^+$ macrophages are located peripherally in the lymphoid structure. Similar distribution of macrophages is observed by other authors [55, 56]. IL-$6^+$ cells are less present in the tertiary lymphoid structure, but are mostly found in alveoli. IL-$6^+$ cells are mainly macrophages and lymphocytes as has been shown by us earlier [57]. The lymphocytic infiltration in alveolar septal wall is inferior with prevalence of $CD3^+$ lymphocytes and less $CD4^+$, $CD8^+$ and $CD20^+$ B lymphocytes. $STAT3^+$ immune cells are numerous and IL-$17^+$ and $FOXP3^+$ immune cells are large in numbers. All lymphocyte subtypes are much more in the peribronchial lymphoid structure. STAT3 in the nucleus is associated with IL-6/JAK/STAT3 pathway [57].

Notably, $CD68^+$ alveolar macrophages are significantly increased and filled alveoli, where detached ATII cells are also seen. $CD68^+$ macrophages are presented in diverse forms– aggregated or diffusely distributed. It is known that the S protein of COVID-19 virus interacts with $CD68^+$ macrophages who engulf and carry the virus [28, 52]. Macrophages are crucial in triggering the "cytokine storm", since they secrete IL-1β, IL-6, IL-10, and TNFα cytokines and chemokines such as CCL2, CCL3 and CCL4 [31, 33]. It has been proven that there are two distinct types of lung macrophages the ones are located between pneumocytes and the others lay in the interstitial alveolar walls and are recruited from blood [28, 58]. Alveolar macrophages are affected by the virus and serve as a first line of defense against it. They provoke an innate immune response. Sars-Cov-2 viruses replicate into these macrophages a phenomenon, called "the macrophage paradox" [50]. They induce the release of pro-inflammatory cytokines, whereas anti-viral cytokine production of IFNγ has been absent [33, 59, 60]. However, viral infection may convert these macrophages into long living cells that can migrate in different tissues [28].

It is obvious that the immune system undergoes profound alterations in COVID-19 disease, including macrophage accumulation and cytokine release in the lung and prevailing lymphocyte infiltration as reviewed by others [61]. The activation of monocytes and dendritic cells after infection by COVID-19 virus results in increased IL-6 secretion (pro-inflammatory cytokine). This triggers the "cytokine storm" that involves the secretion of VEGF, MCP-1, IL-8 and additional IL-6, the latter contributing to increased vascular permeability and leakage [62, 63]. Many other cytokines are elevated in patients with COVID-19, e.g. IL-1β, IL-6, TNFα, IL-12, IFNβ, IFNγ, IL-17 [64]. IFN type I (IFNβ and IFNα) is significantly decreased in severe COVID-19 [65]. The combination of TNFα and IFNγ leads to the induction of inflammatory cell death the so called PANoptosis and following cytokine shock [63, 66]. In COVID-19 patients low levels of IFN type I are observed. Moreover, the reduction of $CD4^+$ and $CD8^+$ T cells, B cells and NK cell counts has been observed [31]. Our immunohistochemical results also show such lymphocytes reduction in alveolar cell walls. The "cytokine storm" is an uncontrolled, cytokine mediated response in viral infections and other conditions. The infected cells through receptor-ligand interactions activate large number of cells including T cells, B cells, macrophages, NK cells, dendritic cells and monocytes [67]. In summary, the antigen-specific and antigen-independent immune activation lead to the development of cytokine storm. IL-6 is the main cytokine that maintains the cytokine storm [68]. It promotes the differentiation of T helpers and $CD8^+$ T cells and inhibits the production of FoxP3 regulatory T cells [31]. In our case we show small numbers of T and B cells and increased number of macrophages in lung that can explain the pathogenesis of alveolar changes in COVID-19 in the infant.

SARS-CoV-2 coronavirus enters target cells via angiotensin-converting enzyme 2 (ACE2) receptor and via transmembrane serine protease 2 (TMPRSS2) [69]. ACE2 downregulation activates the angiotensin II/angiotensin receptor (AT1R) hypercytokinemia [70]. Moreover, SARS-Cov-2 infects cells that have Fc receptors (FcRs) such as macrophages, monocytes and B cells [71]. In our case, SARS-CoV-2 infection exhibits severe respiratory failure, characteristic chest CT scan signs, pneumonia, pulmonary edema and ARDS with aggressive inflammatory responses as reported early [72]. The virus can enter dendritic cells and macrophages via patern recognition recetors (PRRs), namely toll-like receptors (TLRs) such as TLR2, TLR4 and TLR6, that recognize structurally conserved molecules derived from microbes [69], a process that initiates antigen presentation to local naïve T cells and triggers the secretion of robust amount of cytokines and chemokines [73–75]. IL-6 is a major pro-inflammatory cytokine, that through activation of JAK/STAT3 pathway stimulates genes that promote epithelial cell hyperplasia, suppresses genes encoding cell apoptosis, promotes T cell proliferation, activating Th2 differentiation, promoting Th17 cell proliferation and suppressing Treg production [57, 74, 76]. Furthermore, Tregs are decreased in Covid-19 patients [77], a fact that increases the severity of the cytokine storm. Our finding is that $FOXP3^+$ Tregs are moderately presented in lung alveoli. The same is true for $Th17^+$ T cells, induced by IL-6 in lung tissue. $Th17^+$ cells aggravate the cytokine storm releasing more and more inflammatory cytokines such as IL-17 A, IL-17 F, IL-21, and IL-22 [78]. Numerous macrophages and monocytes in alveoli and septa of our case secrete large amounts of IL-6, other cytokines

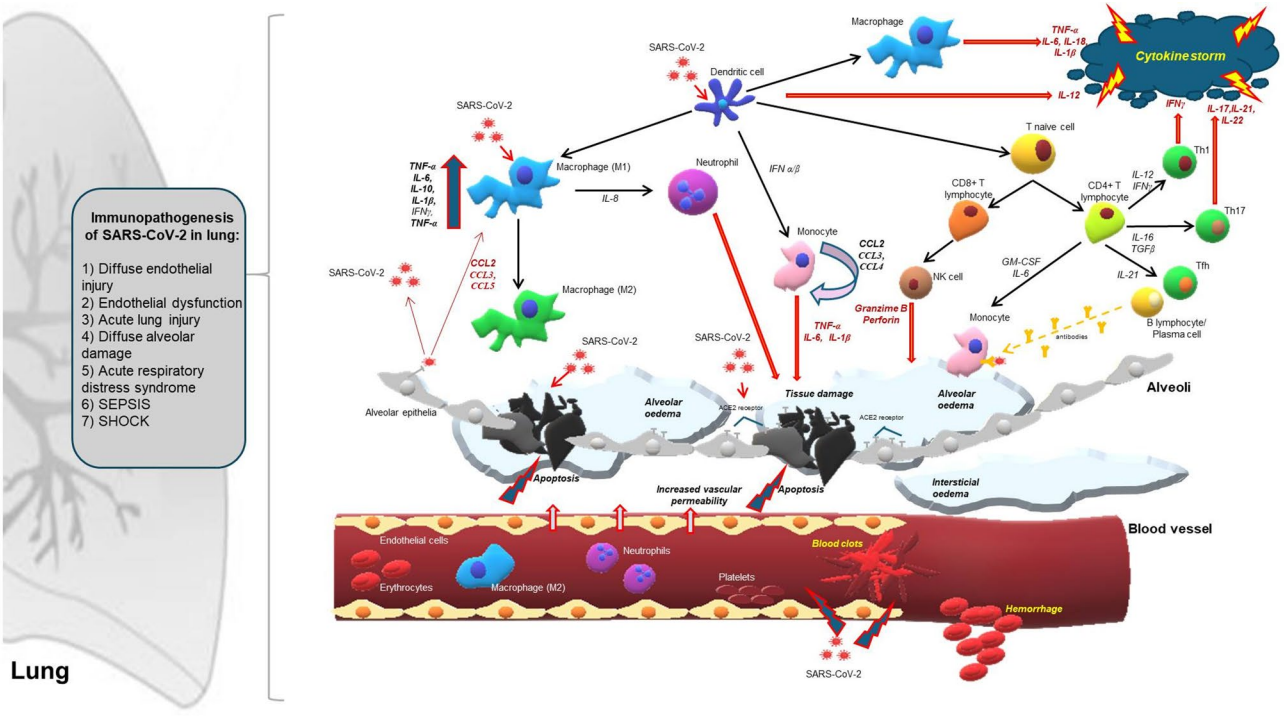


**Fig. 9** Immunopathogenesis of SARS-CoV-2 in lung, modification. SARS-CoV-2 coronavirus enters target cells via angiotensin-converting enzyme 2 (ACE2) receptor and via transmembrane serine protease 2 (TMPRSS2). Moreover, SARS-Cov-2 infects cells that have Fc receptors (FcRs) such as macrophages, DCs, NK cells and B cells. IL-6 is a major pro-inflammatory cytokine, that through activation of JAK/STAT3 pathway stimulates genes that promote epithelial cell hyperplasia, suppresses genes encoding cell apoptosis, promotes T cell proliferation, activating Th2 differentiation, promoting Th17 cell proliferation and suppressing Treg production. Furthermore, Tregs are decreased in Covid-19 patients, a fact that increases the severity of the cytokine storm. Our finding was that FOXP3$^+$ Tregs were moderately presented in lung alveoli. The same was true for Th17$^+$ T cells, induced by IL-6 in lung tissue. Th17$^+$ cells aggravate the cytokine storm releasing more and more inflammatory cytokines such as IL-17 A, IL-17 F, IL-21, and IL-22. Numerous macrophages and monocytes in alveoli and septa of our case secrete large amounts of IL-6, other cytokines IL-1β, IL-10, and TNFα and chemokines such as CCL2, CCL3 and CCL4. Dendritic cells secrete also IL-6 and IL-12, while Th1 cells secrete IFN-γ. The cytokine-dependent immune amplification sustains hyperinflammation of Covid-19. Thus, the initial secretion of IL-6 by infected lung pneumocytes type II attracted massive infiltration of immune cells and release of pro-inflammatory cytokines and chemokines, which further increase the cytokine levels in severe stages of Covid-19 lung injury. (Adapted by Yun-yu Zhang et al. (2020) and Abubakar Umar Anka et al. (2020))

IL-1β, IL-10, and TNFα and chemokines such as CCL2, CCL3 and CCL4 [31, 33, 70]. Dendritic cells secrete also IL-6 and IL-12 [75], while Th1 cells secrete IFN-γ [31]. The hyperactivation of nuclear factor-kappa B (NF-κB) is mediated by large quantities of secreted IL-6 is known as mechanism called IL-6 amplifier, that causes IL-6 hypersecretion in and around non-immune cells [67, 69]. The cytokine-dependent immune amplification sustains hyperinflammation of Covid-19 [67]. Thus, the initial secretion of IL-6 by infected lung pneumocytes type II attracted massive infiltration of immune cells and release of pro-inflammatory cytokines and chemokines, which further increase the cytokine levels in severe stages of Covid-19 lung injury [31, 62, 67, 71]. (Fig. 9)

Our morphological results including increased IL-6 secretion and STAT3 positivity in the lung that trigger the "cytokine storm" could justify the application of IL-6 receptor inhibitor Tocilizumab in children, although its rare application [79].

Our study has some limitations. We present only one case of an infant died from COVID-19 studied morphologically. Since autopsies during pandemic are not allowed, we couldn't study more cases. The advantages of our investigation are morphological characteristics of pneumocytes type II (we show three markers typical for them) and describe them electron microscopically. We show the main immune cells in COVID-19 pneumonia i.e. alveolar macrophages, T and B lymphocytes, and cells expressing IL-6 and STAT3 (member of IL-6 signaling pathway). We present short explanation of the contemporary information about the "the cytokine storm". In our opinion SARS-CoV-2 is a virus that vigorously stimulates immune cells and is one of the rare microorganisms that trigger the appearance of TLS in the lungs.

At the end we want to answer the question why the children are less affected. Children generally experience infrequent, mild, and self-limiting infections which may be due to: (a) higher levels of cross-neutralizing antibodies; (b) lower levels of ACE2 receptors in lung epithelium;

(c) immature T and B cells [43]. In children IL-6 is well expressed in viral infection and Tregs ($FoxP3^+$) are more frequent. Humoral and cellular immunity are yet not well developed [80].

## Conclusions

In SARS-CoV-2 infection in an 11-month old infant we observe exudative diffuse alveolar damage and interstitial pneumonia. The alveolar damage is presented by expression of ATII hyperplasia and macrophage accumulation. ATII cells are important target of SARS-CoV-2 infection. Lymphocyte infiltration (CD3, CD4, CD8 cells) is moderately expressed and located in alveolar walls. $FoxP3^+$ Tregs, IL-17 T helpers and $STAT3^+$ immune cells are increased in the lung tissue. The intensive expression of STAT3 is a sign of induced IL-6 signaling pathway (IL-6/JAK/STAT3 pathway). Our findings highlight the complexity of COVID-19 lung affection, involving ATII cell hyperplasia, interstitial mononuclear cell infiltration and macrophages increase. The findings provide an additional knowledge on the pathophysiology of COVID-19 in the lung and can serve as a basis for studying the cellular and molecular mechanisms of this disease.

### Abbreviations

| | |
|---|---|
| ACE2 | Angiotensin-converting enzyme 2 |
| ATI | Alveolar type I |
| ATII | Alveolar type II cells |
| CCL | C-C motif chemokine ligand |
| CK-19 | Cytokeratin 19 |
| COVID-19 | Coronavirus disease 2019 |
| IL | Interleukin |
| IFNγ | Interferonγ |
| JAK | Janus kinase |
| MCP-1 | Macrophage chemoattractant protein-1 |
| NF-κB | Nuclear factor-kappa B |
| PCR | Polymerase chain reaction |
| PRRs | Patern recognition recetors |
| SARS-CoV-2 | Severe Acute Respiratory Syndrome-Coronavirus 2 |
| STAT3 | Signal transducer and activator 3 |
| Th2 | T helper 2 |
| Th17 | T helper 17 |
| TLRs | Toll-like receptors |
| TNFα | Tumor necrosis factorα |
| TTF-1 | Thyroid transcription factor 1 |
| VEGF | Vascular endothelial growth factor |

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### Author contributions

MG contributed to the conception of the study; VM contributed to TEM, EM micrographs and information about pneumocytes type II; DC contributed to interpretation of cytokine storm and IL-6; MM and LP contributed to patient's diagnosis (X-ray) and treatment; MG and JA contributed to the discussion and the Fig. 9 and approved the final manuscript.

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### Data availability

All data and materials (paraffin embedded tissues from autopsy) are available in the Clinic of general and clinical pathology, University hospital „Prof. Dr. Stoyan Kirkovich" Stara Zagora, Bulgaria.

## Declarations

### Ethics approval and consent to participate

This manuscript was approved by the Ethics Committee of Medical Faculty, Trakia University, in accordance with the ethical standards of the 1964 Helsinki declaration or comparable ethical standards. Written informed consent was obtained from parents of the infant.

### Consent for publication

Written informed consent is obtained from each patient when he is admitted to the University hospital „Prof. Dr. Stoyan Kirkovich" Stara Zagora, Bulgaria.

### Competing interests

The authors declare that they have no competing interests.

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