BPIFB4 Circulating Levels and Its Prognostic Relevance in COVID-19

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Abstract

Aging and comorbidities make individuals at greatest risk of COVID-19 serious illness and mortality due to senescence-related events and deleterious inflammation. Long-living individuals (LLIs) are less susceptible to inflammation and develop more resiliency to COVID-19. As demonstrated, LLIs are characterized by high circulating levels of BPIFB4, a protein involved in homeostatic response to inflammatory stimuli. Also, LLIs show enrichment of homozygous genotype for the minor alleles of a 4 missense single-nucleotide polymorphism haplotype (longevity-associated variant [LAV]) in BPIFB4, able to counteract progression of diseases in animal models. Thus, the present study was designed to assess the presence and significance of BPIFB4 level in COVID-19 patients and the potential therapeutic use of LAV-BPIFB4 in fighting COVID-19.

BPIFB4 plasma concentration was found significantly higher in LLIs compared to old healthy controls while it significantly decreased in 64 COVID-19 patients. Further, the drop in BPIFB4 values correlated with disease severity. Accordingly to the LAV-BPIFB4 immunomodulatory role, while lysates of SARS-CoV-2-infected cells induced an inflammatory response in healthy peripheral blood mononuclear cells in vitro, the co-treatment with recombinant protein (rh) LAV-BPIFB4 resulted in a protective and self-limiting reaction, culminating in the downregulation of CD69 activating-marker for T cells (both TCD4+ and TCD8+) and in MCP-1 reduction. On the contrary, rhLAV-BPIFB4 induced a rapid increase in IL-18 and IL-1b levels, shown largely protective during the early stages of the virus infection. This evidence, along with the ability of rhLAV-BPIFB4 to counteract the cytotoxicity induced by SARS-CoV-2 lysate in selected target cell lines, corroborates BPIFB4 prognostic value and open new therapeutic possibilities in more vulnerable people.

Keywords: Immunity function, Longevity, Plasma, SARS-CoV-2

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causing COVID-19 pandemic is now well known to be a Coronaviridae family virus which spreads quickly in the whole human population through direct contact and aerosol transmission among people in close contact. The wide-ranging symptoms have made the understanding of the epidemiological potential of COVID-19 very challenging for the scientific community. COVID-19 patients can be categorized as asymptomatic, mild/moderate, or severe cases. Acute respiratory distress syndrome and multiple-organ failure characterize high severe
COVID-19 cases in addition to cough, fever, fatigue, myalgia, dyspnea, and bilateral lung infiltration that are also peculiar of milder forms of the disease (1,2). In addition, disseminated intravascular coagulation, leading to acute cardiac injury and pulmonary embolism, is commonly observed in severe cases and is strongly associated with mortality (2,3).

A growing number of data highlighted that SARS-CoV-2 infection overly boosts the immune system leading to a “cytokine storm” (4,5). After a proteolytic processing of the spike protein, SARS-CoV-2 binds ACE2 receptor expressed in host nasal epithelial cells, lungs, and bronchial branches. Following the membrane fusion and viral endocytosis, SARS-CoV-2 starts to replicate in the host cells and triggers an uncontrolled immune response associated with a defective virus clearance and an inflammatory imbalance. The high-level activation of immune cells results in a high production of inflammatory cytokines (ie, IL-6, IL-2R, IL-7, IL-8/CXCL8, IP10, MCP-1/CCL2, MIP1A/CCL3, TNF-α) that encounters a lack of anti-inflammatory pattern required to fine tune the cytokine-driven response, eliciting acute lung damage and fatal multiple-organ failure (6).

Host co-factors (ie, old age, comorbid chronic conditions) hold the key to establish the severity of COVID-19 infection outcome. It is not surprising that old people are the most vulnerable to COVID-19 seen that aging is notoriously related to a low-grade chronic inflammation (ie, inflamming) (7) that drives a frailty condition, which may become breeding ground for the COVID-19 onset (8,9). In support, a recent meta-analysis highlighted that the increase in clinical frailty score was positively associated with the increase of mortality outcome in old patients with COVID-19 (10).

Studies in long-living individuals (LLIs) clearly indicated that, in spite of their extreme chronological age, they are protected from and cope better with age-related diseases, mainly cardiovascular ones (11). This led to hypothesize that LLIs may show a better response to environmental challenges, such as SARS-CoV-2 infection (9). Accordingly, a degree of resiliency of male centenarians has been recently registered in the Lombardy region, Italy (12).

In this scenario, the immune asset and circulating soluble factors that characterize LLIs may offer new disease biomarkers and therapeutic opportunities in COVID-19 pandemic.

The bactericidal/permeability-increasing fold-containing family-B-member-4 (BPIFB4) protein was found being particularly abundant in respiratory secretion, upper airways and proximal trachea (13,14). Noteworthy, elevated level of this innate immunity belonging protein also selectively marks the plasma of healthy LLIs compared to frail ones (15,16). This allowed us to speculate a protective role against cardiovascular effects of aging. Indeed a longevity-associated pattern required to fine tune the cytokine-driven response, eliciting the lysate-induced cytotoxicity and counterbalancing the recombinant LAV-BPIFB4 (rhLAV-BPIFB4) ability in counteracting the lysate-induced cytotoxicity and counterbalancing the pro-inflammatory reaction following the infection.

Method

Patient Recruitment

A cohort of 171 individuals has been recruited to perform the study: n = 49 LLIs (age > 95; median age 96, range 95–99) constituting the control group for the BPIFB4 levels dosage; n = 58 SARS-CoV-2-negative individuals (median age 64, range 24–81); n = 64 SARS-CoV-2-positive individuals (median age 65, range 20–91). For each individual, detailed anamnesis and plasma from venous blood was collected for the analyses. All participants signed an informed consent for the management of personal anamnestic data and blood samples. The SARS-CoV-2-positive group had received a diagnosis of COVID-19 based on a positive naso-pharyngeal swab for SARS-CoV-2 RNA. Peripheral blood samples were collected from each patient within 7 days from the admission to the Infectious Diseases Unit of “Giovanni di Dio e Ruggi d’Aragona” University Hospital of Salerno. Clinical laboratory analyses testing at hospital admission included the following: complete blood count (leucocytes, lymphocytes, platelets), mean corpuscular volume, hematocrit, hemoglobin, erythrocyte sedimentation rate, LDH, serum ferritin, D-dimer, CRP, and fibrinogen (Supplementary Table 1). Patients were stratified in 2 groups according to needing of oxygen or ICU admission. Thirty-two patients (low grade) with oxygen saturation between 90% and 94% that did not need ICU admission were considered as having mild–moderate COVID-19. Thirty-two patients (high grade) with an oxygen saturation below 90% at admission or during the hospital stay that required either noninvasive or mechanical ventilation or need of admission to the ICU were considered as having severe COVID-19.

The study was approved by the IRCCS MultiMedica and by the internal Ethics Committee of “Giovanni di Dio e Ruggi d’Aragona” University Hospital of Salerno and conducted in accordance with the ethical principles deriving from the Declaration of Helsinki.

Cell Lines and Culture Condition

Adenocarcinomic human alveolar basal epithelial cells (A549 cells, ATCC CCL-185) were grown in a humidified incubator at 37°C and 5% CO2 in DMEM (Gibco, Thermo Fisher Scientific) supplemented with 10% (v/v) fetal serum bovine (FBS, Gibco, Thermo Fisher Scientific), 1% (v/v) penicillin–streptomycin (Aurogene), 1% (v/v) MEM non-essential amino acids (MEM NEAA, Gibco, Thermo Fisher Scientific), and 1% (v/v) sodium pyruvate (Aurogene). Human bronchial epithelial cells (BEAS-2B, ATCC CRL-9609) were grown in a humidified incubator at 37°C, 5% CO2 in DMEM/F12 (Lonza BioWhittaker) supplemented with 15% FBS.
490 nm was measured using a microplate reader. Conditioned media collected as above mentioned. Absorbance at 37°C in the assay-coated microplate. After removing any unbound substances, a biotin-conjugated antibody specific for C20orf1186 was added to the wells and incubated for 1 hour at 37°C. After washing, avidin-conjugated horseradish peroxidase (HRP) was added to the wells and incubated for 1 hour at 37°C. Following a wash, substrate solution was added and the consequent color development was stopped. Optical density was measured at 450 nm.

Cytokines Detection

Cytokines levels in PBMCs-conditioned media, BEAS-2B, and HUVECs supernatants were determined using a beads-based multiplex ELISA (LEGENDplex, Biolegend, USA). Conditioned media were incubated for 2 hours with the beads and for 1 hour with the detection antibodies, followed by 30 minutes incubation with SA-PE. After washing, beads were resuspended in washing buffer and acquired using a FACS VERSE flow cytometer (BD Biosciences). Data were analyzed with the LEGENDplex Data Analysis Software.

Antibodies and Flow Cytometry

PBMCs (treated as above mentioned) were stained with mAb against human CD3 PerCP-Cy5.5 (Biolegend #300327, 5 μL/test) and CD69 FITC (Miltenyi Biotec, 5 μL/test) CD4 PE-Vio770 (Miltenyi Biotec, 2 μL/test), and CD8 APC-Vio770 (Miltenyi Biotec, 2 μL/test). After 30 minutes incubation at 4°C in the dark, cells were washed, centrifuged, and resuspended in staining buffer for the FACS analysis. For each test, cells were analyzed using FACS Verse Flow Cytometer (BD Biosciences).

Statistical Analysis

In all experiments shown, statistical analysis was performed by using the GraphPad prism 6.0 software for Windows (GraphPad software). For each type of assay or phenotypic analysis, data obtained from multiple experiments are calculated as mean ± SD and analyzed for statistical significance using appropriate tests. In analysis of variance (ANOVA) for multiple comparison, p values < .05 were considered significant; *p < .05, **p < .01, and ***p < .001.

Results

BPIFB4 Blood Levels Are Decreased in COVID-19 Patients

BPIFB4 has been shown to serve as a biomarker of healthy aging (15,16) and previous finding on its prognostic significance in vascular pathology (ie, atherosclerotic patients) (18), prompted us to examine BPIFB4 contribution in COVID-19. We examined the plasma BPIFB4 levels in n = 64 patients with COVID-19 (median age 65, range 20–91) consecutively admitted to “San Giovanni di Dio e Ruggi d’Aragona” University Hospital of Salerno. Clinical and laboratory features of COVID-19 patients are shown in Supplementary Table 1. For comparison, both a first cohort of n = 49 LLIs (age > 95; median age 96, range 95–99) and a second cohort of n = 58 SARS-CoV-2-negative individuals (median age 64, range 24–81) constituted the 2 control groups for the BPIFB4 levels dosage (Figure 1).

Importantly, BPIFB4 values were significantly lower in SARS-CoV-2-positive individuals as compared with SARS-CoV-2-negative ones (57.31 ± 53.13 pg/mL vs 108.1 ± 66.4 pg/mL, p = .0002) pointing to BPIFB4 as a bona fide biomarker inversely related to COVID-19 diagnosis. In parallel, we confirmed that LLIs have...
higher levels of BPIFB4 as compared to old healthy controls (179.80 pg/mL ± 100.4 vs 108.1 ± 66.4 pg/mL; p < .0001; Figure 1).

Based on previous studies on the prognostic relevance of BPIFB4 in vascular pathology and its association with the degree of carotid stenosis in atherosclerotic patients (18), we moved to analyze the plasma BPIFB4 levels in n = 32 severe (high grade) and n = 32 nonsevere (low grade) COVID-19 patients. In our cohort of n = 64 COVID-19 subjects, the high-grade patients presented with oxygen saturation values <90% and needing of oxygen or ICU admission. On the basis of the laboratory test, as previously reported (19,20), we observed higher significant levels in LDH and CRP and reduced platelets count in severe patients with respect to nonsevere ones, indicating inflammatory burst in high-grade group (Figure 2A and B). Of note, the average plasma BPIFB4 level in the severe group (high grade) was significantly lower than in nonsevere group (low grade) (35.91± 45.22 pg/mL vs 71.54 ± 52.84 pg/mL, p = 0.0177; Figure 2D). Taking all these in account, BPIFB4 plasma levels are inversely correlated with disease severity, even though no significant correlations were found between BPIFB4 and other COVID-19 inflammatory and prognostic markers (CRP, D-dimer, ferritin, etc.; data not shown).

Recombinant Human LAV-BPIFB4 Blunts Inflammatory Response to Lysates From SARS-CoV-2-infected Cells in PBMCs In Vitro

The reduced plasma level of BPIFB4 in COVID-19 patients led us to deeply investigate the putative protective role of BPIFB4 in vitro studies.

LAV of BPIFB4 gene was described to protect from age-related endothelial dysfunction (21,22) and atherosclerosis, 2 main age-related conditions, mainly by conferring subjects with a favorable maintenance of nitric oxide bioavailability and huge anti-inflammatory profile. Thus, we explored the influence of recombinant LAV-BPIFB4 on the primary response of PBMCs from healthy donors upon stimulation with digested lysates from SARS-CoV-2-infected cells.

In an experimental setting in vitro, we demonstrated an increased CD3+T cell reactivity (both TCD4+ and TCD8+) when healthy PBMCs were pulsed with SARS-CoV-2 lysates from 48 to 72 hours. Indeed, a significant higher percentage of CD69+ activated lymphocytes were found among SARS-CoV-2 lysates-treated PBMCs as compared to nontreated ones both at 48 hours (9.2 ± 1.7 vs 3.9 ± 0.7; p = 0.05 for TCD8+ cell subset and 5.1 ± 0.8 vs 3.7 ± 0.4; p = 0.05 for TCD4+ cell subset) and soon after at 72 hours (16.2 ± 2 vs 6.2 ± 0.8; p = 0.004 for TCD8+ cell subset and 10.1 ± 1.8 vs 6.5 ± 0.9; p = 0.004 for TCD4+ cell subset). When PBMCs exposed to SARS-CoV-2 lysates were pretreated with rhLAV-BPIFB4, the expression of CD69 activation marker was modulated in a time-dependent manner. Specifically, at the early time points (48 hours after SARS-CoV-2 lystate burst), the treatment with rhLAV-BPIFB4 induced an increase in the percentage of both CD69+TCD8+ (13.9 ± 1.9 vs. 9.2 ± 1.7; p = .0001) and CD69+TCD4+ (13.3 ± 2.1 vs 5.1 ± 0.8; p = .0001), establishing a primary line of defense. Later, 72 hours after SARS-CoV-2 lystate burst, rhLAV-BPIFB4 treatment significantly reduced the expression of CD69 activation marker on the surface of both TCD8+ (8.9 ± 1 vs 16.2 ± 2 vs. 6.2 ± 0.8; p = .0021) and TCD4+ (5.9 ± 0.78 vs. 10.1 ± 1.8; p = .0021; Figure 3A and B). Further, as most of the LAV-BPIFB4 immunomodulatory effects are related to myeloid compartment, we performed experiments of PBMCs activation in absence of CD14+ monocytes in order to verify if the latter are also determinants for LAV-BPIFB4 effect in response to SARS-CoV2 lystate stimulation. As expected, under the above conditions, ie the peripheral blood lymphocytes alone, rhLAV-BPIFB4 was not able to increase the percentage of both CD69+TCD4+...
and CD69+TCD8+ at 48 hours, neither to reduce the expression of CD69+ activation marker on the surface of both T cell subsets later at 72 hours (Figure 3B). These results may point to a unique role of LAV-BPIFB4 in inducing a first protective response and then in reducing the magnitude of lymphocyte response upon persistent inflammatory stimuli. As consequence LAV-BPIFB4 treatment had also an effect on cytokine release in the virus lysate pulsed-PBMCs. As shown in Figure 3C, a specific MCP-1 production was observed in PBMCs in response to stimulation by SARS CoV-2 lysate compared with control PBMCs pulsed with cell lysate alone (1989 ± 456 pg/mL vs 1367 ± 221 pg/mL) or compared with nonpulsed PBMCs (1989 ± 456 pg/mL vs 1201 ± 112 pg/mL; p = .043). Consistent with its immunomodulatory role, we found that the pretreatment with rhLAV-BPIFB4 (18 ng/mL) significantly reduced MCP-1 release (790 ± 124 pg/mL vs 1989 ± 456 pg/mL; p = .011), which is responsible for abundant inflammatory cell infiltration to sites of infection in vivo.

It is well established that an imbalanced host immune response to SARS-CoV-2 drives COVID-19, as not only pro-inflammatory cues but also low innate antiviral defenses may contribute to disease development. Thus, we also tested the LAV-BPIFB4 effect on the secretion of the inflammasome-related cytokines IL-1β and IL-18, described as largely protective during murine coronavirus infection in vivo (23). Interestingly, SARS-CoV-2 lysate-pulsed PBMCs when preliminary exposed to LAV-BPIFB4, secreted significantly more IL-1β (Figure 3D) and IL-18 (Figure 3E) than non-pretreated PBMCs (1705 ± 234 vs 66 ± 12 pg/mL; p = .0001 for IL-1β and 241 ± 19 vs 18 ± 32 pg/mL; p = .0001 for IL-18), as determined by multiplex ELISA.
LLIs, by maintaining a fine balance between inflammatory and anti-inflammatory circuits, age slowly and healthy, avoiding chronic cardiovascular diseases. Accordingly, while mortality increases up to very old ages, a resilience of men older than 90 years was documented in the North of Italy (12). Furthermore, clinical and epidemiological data from Cilento, a rural area in the Southern of Italy mainly enriched of nonagenarians and centenarians, indicated that LLIs develop more resiliency to COVID-19, in term both of the occurrence of pathological event and a better tendency to illness’ recovery (personal communication).

In this scenario, the immune asset and soluble factors characterizing the long lifespan of LLIs may offer new diagnostic and therapeutic opportunities in COVID-19 pandemic.

The LAV of the bactericidal/permeability-increasing fold-containing-family-B-member-4 (BPIFB4) has been associated with exceptional longevity, and gene therapy with this isoform was able to improve revascularization and endothelial function (21). Furthermore, atherosclerotic process was blunted by LAV-BPIFB4 in a mouse and ex vivo human model (18); at the same way, diabetic cardiopathy was attenuated by LAV-BPIFB4 gene transfer in mice model of disease (25). These effects can be in part explained by the LAV-BPIFB4 ability to influence the polarization of human and murine monocytes by tuning their differentiation process toward dendritic cells with regulatory functions (IL-10 and TGF-beta producing cells) in human healthy donors (14) and toward pro-resolving M2 macrophages in ApoE/-/- mice (18), also by positively affecting the SDF-1/CXCR4 axis (18,25). To be noted, BPIFB4 is a secreted protein belonging to the BPI/lipopolysaccharide-binding protein (LBP) family of antibacterial components that participates in host protection through antimicrobial and surfactant properties at the upper airways (13). A recent study showed that the ACE2-enriched mucosa of oral cavity can be responsible for the virus easy access to a new susceptible host (26) and underpins the importance of full competent cellular and humoral artillery to cope with the virus spreading.

We have recently described that BPIFB4 levels are increased in plasma and PBMCs of LLIs (15,27), in CD34+ cells of LLIs (21) and in serum of healthy versus non-healthy (frail) LLIs (16) and are closely related to a balanced immune response both dampening the deleterious activation and counterbalancing the immune decline in steady state.

The hypothesis is that BPIFB4, originally belonging to a family of host defense proteins, (28), may be a new longevity-associated determinant of COVID-19 patients. Our observational clinical study on n = 64 COVID-19 patients helped to assess the predictive value of blood level of BPIFB4 of COVID-19.

Indeed the reduced circulating levels of BPIFB4 in patients with severe disease (characterized by elevated levels of CRP and LDH) validate its prognostic significance for severity in patients with COVID-19 (Figures 1 and 2). This is in agreement with previous findings by our group highlighting the importance of BPIFB4 levels to classify both the health status of LLIs (by discriminating frail individuals vs non-frail ones) (16) and to predict the atherosclerotic risk being the protein’s concentration significantly higher in subclinical carotid atherosclerosis and in patients with IMT <2 mm, as compared to patients with carotid stenosis (18).

Of note, the lack of correlations between circulating concentrations of BPIFB4 and other inflammatory markers (CRP, LDH, ferritin, D-dimer, lymphocyte count, etc., data not shown) may be consistent with the hypothesis that the low levels of BPIFB4 in high-grade COVID-19 patients may represent at most the cause and not the consequence of the disease. It is unlikely that low level
of BPIFB4 in high severity group may be related to its consumption by the high inflammatory background; rather we hypothesize that based on genotype, or the peculiar stability of the protein, the high circulating level of BPIFB4 characterizing some COVID-19 patients, may induce a homeostatic response and a better tendency to the high inflammatory background as seen in the context of “inflamming” of old individuals. Indeed, as LILs that carry high levels of BPIFB4, are able to better cope with disabilities linked to inflammatory conditions, at the same way high level of BPIFB4 can blunt the inflammatory burst typical of the COVID-19.

The functional role of the circulating BPIFB4 was magnified when examining its contribution to properly tune the inflammatory response of healthy PBMCs to SARS-CoV-2 lysozyme (Figure 3). In a monocyte-dependent manner, LAV-BPIFB4 was able to induce a distinct T cell response upon SARS-CoV-2 lysozyme stimulation, described to be both protective (48 hours) and self-limiting (72 hours; Figure 3A and B), that would be relevant in conferring protection from the “cytokine storm” to which several tissues, such as bronchial cells (Supplementary Figure 1B), are exposed in COVID-19.

Indeed, in a second experimental setting in vitro, we confirmed the protective role of high protein levels of BPIFB4 by decreasing MCP-1 responsible for hyper-innate inflammatory response in SARS-CoV-2 infection through the recruitment of monocytes/macrophages and neutrophils in vivo (29). Again, the finding is in line with the observed reductions of CD68-positive cells at the aortic arch level and of MOMA-2 mono-macrophage-positive cells in femoral arteries of ApoE knockout mice after systemic LAV-BPIFB4 gene therapy using an adeno-associated viral vector (18). The well-established effect of BPIFB4 protein on the mono-macrophage compartment was also highlighted by the peculiar redistribution of monocyte pool, which uniquely marks LILs. As documented elsewhere, circulating levels of nonclassical CD14+CD16+ patrolling monocyte (PMo) were found significantly higher in LILs compared to young and old controls (27). Patrolling monocytes actively patrol the resting vasculature to remove injured cells in a number of chronic conditions where they have been described to assist wound healing process and the resolution of inflammation (30). This functional association together with the degree of protection from hypertension, ischemia and atherosclerosis offered by LAV-BPIFB4 treatment, could in part explain the reduced occurrence of chronic cardiovascular diseases in LILs, endorsing them also with the capacity to better recover after SARS-CoV-2 infection, too.

This is confirmed by the ability of rhLAV-BPIFB4 to limit cellular damage in vitro after SARS-CoV-2 lysozyme (Figure 4) probably mediating a cellular homeostatic response to stress, as already documented for STHdh Q111/111 striatal cells exposed to cytoxic insult of an expanded CAG repeats encoding a long polyglutamine tract in the huntingtin protein (Htt) (31). Indeed, a stress response process was facilitated by the LAV-variant consisting in BPIFB4 phosphorylation/activation by stress kinase protein kinase R-like endoplasmic reticulum kinase PERK, its complexes with 14-3-3 and HSP 90 and calcium/endothelial nitric oxide synthase activation (20–22). The NLRP3 inflammasomes, a complex of multiple proteins that activates the secretion of the proinflammatory cytokine (eg, IL-1β and IL-18) in a caspase-1-dependent manner, often participate in the regulation of an optimal endoplasmic reticulum stress response (ERS) in the injured cell. However, while a transient protective ERS restores protein homeostasis by activating the UPR and reducing protein aggregates, long-term or severe ERS can trigger cell dysfunction and death (32, 33). In our opinion, this protective response mediated by LAV-BPIFB4 may also explain the enhancing production of IL-1β (Figure 3D) and IL-18 (Figure 3E), well-known inflammatory cytokines with a variety of immunomodulatory effects, able of leading to both early host protection and damaging pathological events after chronic deleterious perpetuation of inflammatory response. Among protective effects, IL-18 signaling promotes production of interferon gamma and perforin-mediated cytotoxicity (34–36), crucial for viral clearance. At the same way, IL-1 signaling controls viral replication and the induction of a protective T cell response during virus infection (37, 38).

Interestingly, IL-18 was found to be important for host defense, as suggested by the poor survival and elevated viral replication in mice lacking IL-18 during murine coronavirus infection (23).

Even though the cellular mechanism and the regulatory circuits by which LAV-BPIFB4 induces a homeostatic response are still unknown, the relevance covered by the biphasic immune responses (protective and self-limiting) and the fine tuning of cytokines milieu might in part explain the beneficial effect achieved by LAV-BPIFB4 in the context of COVID-19.

In conclusion, even if the associative nature of data does not permit to definitively conclude that the BPIFB4 plasma level is relevant to the COVID-19 prognosis, to better of our knowledge, our present work constitutes the first study to describe a longevity-associated protein discriminating among severity-based stratified COVID-19 patients.

From a translational point of view, a better characterization of BPIFB4 clinical relevance by investigating its expression in damaged tissues or PBMCs obtained from COVID-19 patients will help to validate BPIFB4 as a valuable biomarker for COVID-19 severity. This may be useful in a more accurate stratification of patients, their management and in treatment decision. Furthermore, its dual role on immune compartment and in fruitful stress response to limit cellular damage makes BPIFB4 an attractive therapeutic tool to counteract complications of COVID-19.

### Supplementary Material

Supplementary data are available at The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences online.

Supplementary Figure 1. Cytokine report analysis conducted by multiplex ELISA on the supernatants of BEAS-2B and HUVEC cells after 72 hours of treatment with 50 μg/mL of SARS-CoV-2 Lysate in presence or absence of 18 ng/mL of rhLAV-BPIFB4 (A). In the experiment shown in the lower panel, BEAS-2B cells were stimulated for 72 hours with conditioned medium CM (diluted 1/5) from resting PBMCs, SARS-CoV-2 treated-PBMCs or LAV-BPIFB4-SARS-CoV-2 co-treated PBMCs. Bar graph showing average cytotoxicity (±SD) determined using LDH assay. P-values indicate significance levels comparing average LDH release among different groups (ANOVA). The data clearly suggest that the positive modulation of PBMCs-cytokine milieu by rhLAV-BPIFB4 would confer protection of bronchial cells during the inflammatory response induced by SARS-CoV-2 (B).

### Author Contributions

E.C. designed and conducted the study and coordinated the research team. V.L., F.M., V.M., A.C., and P.D.P. performed laboratory activities. C.Z. and G.F. were involved in virus lysate preparation. A.F., C.S., V.C., T.I., E.D.B., P.P., and C.V. cared for the subjects of the study and evaluation of their health status and reviewed critically the paper. A.A.P. and E.C. performed statistical
analysis and data interpretation and wrote the manuscript. A.A.P. supervised the project in its entirety and provided financial support. All authors approved the final version to be published.

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Conflict of Interest

A.A.P. and C.V. own shares of LGV1 Inc. and have filed a patent. A.A.P. and E.C. are co-inventors of one international patent application. All other authors declare no financial or competing interests that are directly relevant to the content of this manuscript.

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