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Original Research

NLRP3 and AIM2 inflammasomes expression is modified by LPS and titanium ions increasing the release of active IL-1β in alveolar bone-derived MSCs

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Abstract

Periodontitis and peri-implantitis are inflammatory diseases of infectious etiology that lead to the destruction of the supporting tissues located around teeth or implants. Although both pathologies share several characteristics, it is also known that they show important differences which could be due to the release of particles and metal ions from the implant surface. The activation of the inflammasome pathway is one of the main triggers of the inflammatory process. The inflammatory process in patients who suffer periodontitis or peri-implantitis has been mainly studied on cells of the immune system; however, it is also important to consider other cell types with high relevance in the regulation of the inflammatory response. In that context, mesenchymal stromal cells (MSCs) play an essential role in the regulation of inflammation due to their ability to modulate the immune response. This study shows that the induction of NLRP3 and absent in melanoma 2 (AIM2) inflammasome pathways mediated by bacterial components increases the secretion of active IL-1β and the pyroptotic process on human alveolar bone-derived mesenchymal stromal cells (hABSCs). Interestingly, when bacterial components are combined with titanium ions, NLRP3 expression is further increased while AIM2 expression is reduced. Furthermore, decrease of NLRP3 or AIM2 expression in hABSCs partially reverses the negative effect observed on the progression of the inflammatory process as well as on cell survival. In summary, our data suggest that the progression of the inflammatory process in peri-implantitis could be more acute due to the combined action of organic and inorganic components.

Key words: peri-implant disease; inflammation; NLRP3; AIM2; IL-1β; cell signaling.

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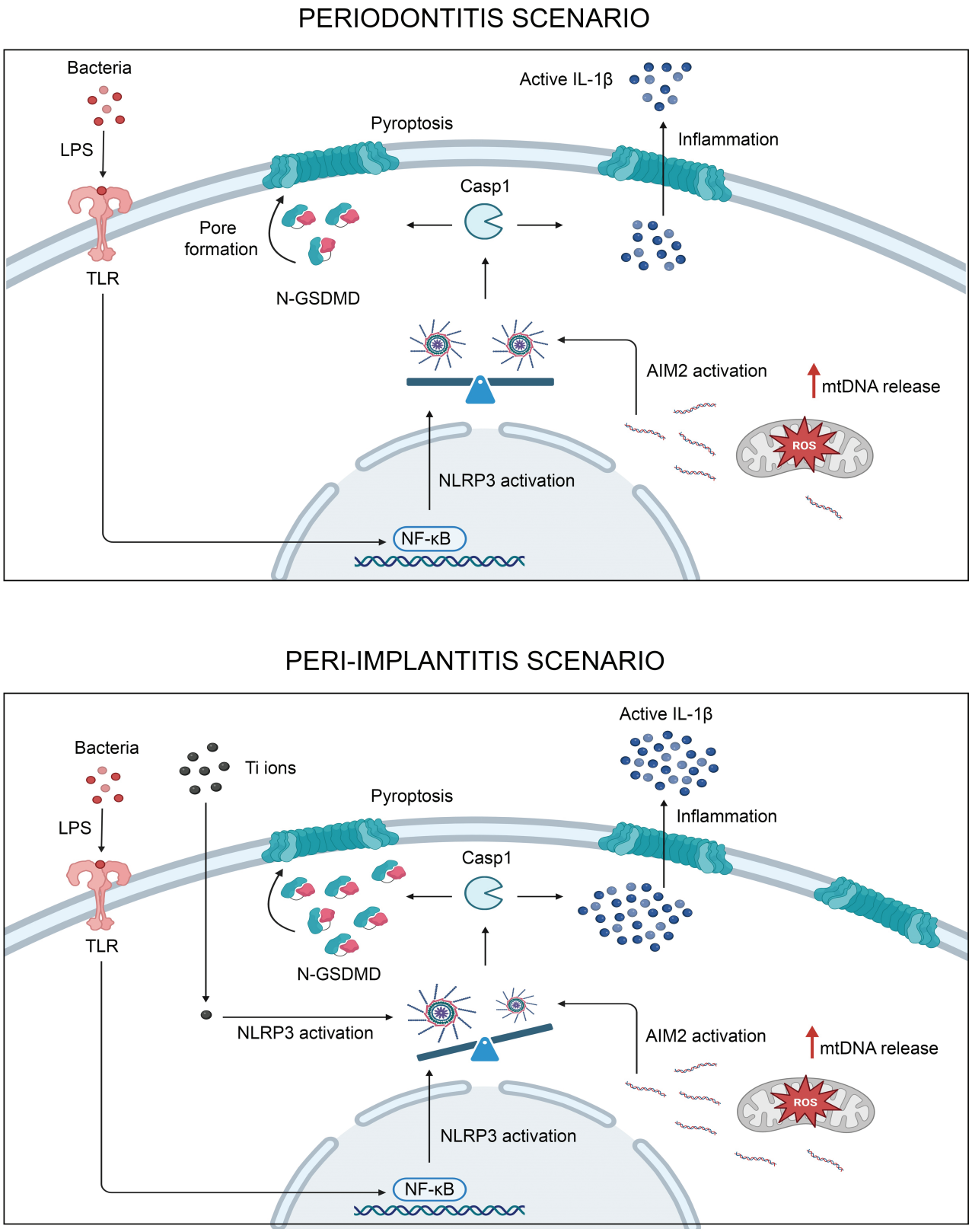
Graphical Abstract

The bacterial component (LPS) has the capacity to induce the expression of NLRP3 and AIM2 inflammasomes, thereby augmenting the secretion of IL-1β by alveolar bone-derived mesenchymal stromal cells (hABSCs) and consequently intensifying the inflammatory process. Furthermore, the coalescence of LPS and metal (titanium ions) exerts a synergistic impact on NLRP3 expression induction, resulting in an amplified inflammatory response.



Significance Statement

This research reveals IL-1β-caspase 1-mediated release from mesenchymal stromal cells derived from maxillary alveolar bone as a consequence of NLRP3 and AIM2 inflammasomes activation promoted by bacterial products as lipopolysaccharides. As a consequence, cell survival is reduced. Interestingly, these phenomena are increased significantly in the presence of titanium ions, but it is inhibited when this inflammasome gene expression is blocked by CRISPR/Cas9 gene edition, which suggests the important role of inflammasomes in the peri-implant inflammation promotion. Furthermore, the relevance that both genes have in cell survival could make them a suitable target to improve the therapeutic efficiency of these cells.

Introduction

Periodontitis consists in a pathological condition associated with dental plaque that normally evolves toward inflammation and histolysis of the supporting tissues that are located around the tooth.[1] It is a highly prevalent disease that affects around 60% of the total adult population.[2] In patients who have a dental piece replaced by an implant, the analog pathology is known as peri-implantitis.[3] Peri-implantitis and periodontitis share several characteristics, such as the bacteria which are responsible for triggering the disease[4] and the molecular signaling pathways responsible for the progression of both pathologies.[5] As far we know, there is a general tendency to believe that the pathophysiological conditions underlying both diseases are the same. However, there is an abundant literature in which it is evident that although those pathologies share the aforementioned characteristics, it is also true that they show important differences.[6] For example, tissue biopsies from patients with peri-implantitis show a greater infiltrate of immune cells compared with those observed in tissue samples from patients suffering from periodontitis.[7] Furthermore, it is also well documented that bone resorption is faster in patients with peri-implantitis.[8]

The release of particles and metal ions from the implant surface could explain, at least in part, the observed differences. Accordingly, our group has previously shown that these released particles can activate BRCA1 and CHK2 genes in oral epithelial cells, which in turn could be the cause of the initiation of the local inflammatory process.[9] The activation of the inflammatory process could also be carried out by the activation of the inflammasome pathway.[10] Inflammasomes are multiprotein complexes belonging to the innate immune system that promote proteolytic cleavage, maturation and secretion of proinflammatory cytokines interleukin-1β (IL-1β) and 18 (IL-18), as well as the cleavage of Gasdermin-D (GSDMD) that induces the formation of pores in cell membranes triggering a type of programmed cell death called pyroptosis. Several types of inflammasomes have been identified in recent decades including NLRP3 (NLR family pyrin domain containing 3) inflammasome that recognizes both organic (bacteria, viruses, etc.) as well as inorganic (uric acid, ATP, or metal particles) components.[11] In turn, absent in melanoma 2 (AIM2) inflammasome only recognizes and is activated by double-stranded DNA (dsDNA).[12]

Inflammasomes are mainly expressed in cells of the innate immune system although they are also expressed in other cell types, such as endothelial, epithelial, or mesenchymal stromal cells.[13-15] Mesenchymal stromal cells (MSCs) are nonhematopoietic multipotent cells present in virtually all tissues and organs. In an inflammatory environment, these cells are essential due to their immunoregulatory and tissue regenerative capacities.[16] Few studies have addressed in vitro the activation of NLRP3 in response to potential triggers of peri-implantitis such as metal particles and ions, and furthermore, these studies have only been carried out in macrophages and T cells.[17,18] However, it is still unknown what role metal ions may play in the activation of the NLRP3 pathway in immunoregulatory cells (MSCs). There are also no previous studies on the role of AIM2 in MSCs when they are located in an inflammatory environment.

Accordingly, the aim of this study was to evaluate the expression of NLRP3 and AIM2 in human alveolar bone-derived mesenchymal stromal cells (hABSCs) in the presence of bacterial components and/or metal ions as well as other characteristics of MSCs such as cell survival. Finally, the consequences of decreasing NLRP3 or AIM2 expression in hABSCs were also analyzed.

Materials and methods

Human alveolar bone-derived mesenchymal stromal cells (hABSCs) collection and isolation

Bone tissue from different donors was collected at the dental clinics of the School of Dentistry (University of Granada). hABSCs were collected from bone during implant placement as previously described.[19] hABSCs were cultured in Regular Dulbecco's modified Eagle medium with 1 g/L glucose (DMEM-LG; Gibco), 10% fetal bovine serum (Sigma-Aldrich), 1:100 of nonessential amino acid solution (Gibco), 0.01 μg/mL of basic fibroblast growth factor (PeproTech), 100 U/mL of penicillin/streptomycin, and 0.25 μg/mL of amphotericin B and were incubated at 21% O_2/5% CO_2 at 37 °C. All hABSCs used in this study were $CD90^+$, $CD73^+$, $CD105^+$, $CD14^-$, $CD34^-$, $CD45^-$, and $CD31^-$. They were also able to differentiate into adipocytes, osteocytes, and chondrocytes (Supplementary Figure S1).

hABSCs treatments

In order to induce NLRP3 and/or AIM2 expression in hABSCs, 0.1×10^6 cells were seeded in 12-well plates. The following day, cells were treated with 1 μg/mL of lipopolysaccharide (LPS *E.coli* O111:B4, Sigma-Aldrich) for 24 hours with or without 20 μg/mL of titanium ions (Ti) solution (titanium atomic absorption standard solution, Sigma-Aldrich). Cells supernatants were subsequently stored at −20 °C for further enzyme-linked immunosorbent assay (ELISA) analysis and TRIzol Reagent (Invitrogen) was directly added onto the cells and stored at −80 °C for subsequent RNA extraction.

Quantitative PCR

Total RNA was isolated using TRIzol reagent according to manufacturer's instructions. RNA samples were reverse transcribed using the PrimeScript RT master mix (Perfect Real Time; TaKaRa Bio Inc.) and quantitative PCR (qPCRs) were performed using the TB Green Premix Ex Taq (Tli RNase

H Plus; TaKaRa Bio Inc.) on a Real-Time PCR Thermal Cycler qTOWER3 system. The primers used are shown in Supplementary Table S1.

Immunofluorescence

For immunofluorescence analysis, 20 000 cells were seeded in 24-well plates and the same treatment with LPS and Ti solution explained above was carried out. After cell incubation with the different reagents, cells were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich), permeabilized with 0.25% Triton X-100 (Sigma-Aldrich), blocked with 2% BSA (Bovine Serum Albumin; Sigma-Aldrich) and subsequently incubated overnight with primary anti-human NLRP3 or AIM2 antibodies (Invitrogen and MyBioSource, respectively). The next day, a secondary antibody (Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, from Invitrogen) was added to the cells for 1 hour and Hoechst was used for nuclear counterstaining. Incubations with only primary or secondary antibodies were also carried out as controls. Images were acquired on a Nikon eclipse Ts2 microscope. Fluorescence intensity was measured using the ImageJ digital image processing program.

Enzyme-linked immunosorbent assay

Collected supernatants were centrifuged at 1000 × *g* at 4 °C for 10 minutes. IL-1β protein levels were measured using the human IL-1 beta uncoated ELISA kit (Invitrogen) according to the manufacturer's protocol. Absorbance was evaluated at 450 nm on an Infinite M200 Pro Microplate Reader and protein concentration was calculated according to a standard curve.

Genome editing of hABSCs

Generation of NLRP3 or AIM2-KO hABSCs was carried out using the CRISPR/Cas9 system. For it, lentiviral vectors (LVs) encoding a guide RNA (gRNA) specific for each gene, as well as the Cas9 protein were used. The CRISPOR tool (CRISPOR.org) was used in order to design 3 different gRNAs for each gene. The sequence of each gRNA is shown in Supplementary Table S2. The final LVs plasmids with the different gRNAs were supplied by Vector Builder Company (VectorBuilder Inc.). An LV plasmid encoding for a nonspecific gRNA was used as control.

Production of LVs and virus titration

Lentiviral particles were produced by polyethyleneimine (Sigma-Aldrich) as previously described.[20] Briefly, 293T packaging cells were cotransfected with (a) specific Cas9 LV, (b) packaging plasmid pCMvdR8.91, and (c) envelope plasmid pMD2.G.47 (VsV-g). Viral supernatants were collected after 24, 48, and 72 hours and filtered through 0.45-µm filters (Nalgene). The viral particles were then concentrated by ultracentrifugation at 90 000 x g for 2 hours at 4 °C in a Beckman Optima Centrifuge (Beckman Coulter).

Viral titers were determined by RT-qPCR using the Stratagene MX30005Pro real-time thermal cycler (Agilent Technologies). Briefly, genomic DNA of K562 cells transduced with different amounts of LVs was isolated (10^5 cells, equivalent to 0.6 g of genomic DNA) and de copy number of integrated LVs was measured using a standard curve (from 10^5 to 10 copies) of plasmid DNA. The primers used for titration were DU3 FW: GACGGTACAGGCCAGACAA) and PBS Rev: TGGTGCAAATGAGTTTTCCA.

Transduction of hABSCs

Transduction of hABSCs was performed as previously described.[21] Briefly, 0.7×10^6 hABSCs were mixed with the concentrated viruses (MOI = 50), left at room temperature for 10 minutes, and subsequently seeded in sex-well plates and incubated at 21% O_2/5% CO_2 at 37 °C. Five hours later, cells were washed and seeded in T75 flasks at 21% O_2/5% CO_2 at 37 °C for 3-4 days. Then, transduced hABSCs were treated with 0.5 µg/mL of puromycin (Sigma-Aldrich) for 1 week to select those cells that had incorporated the vector.

Verification of CRISPR gene editing efficiency

To check the efficiency of gene editing, genomic DNA of transduced and non-transduced (NT) hABSCs was extracted using a Quick-DNA Miniprep Kit (Zymo Research). Genomic regions flanking the CRISPR/Cas9 target site for each gRNA were amplified by PCR with MyTaq Red Mix, 2X Kit (Bioline). The primers used are shown in Supplementary Table S2. PCR products were subsequently purified using the DNA Clean & Concentrator-5 Kit (Zymo Research) and Sanger-sequenced using the same primers that were used to perform the PCR. Finally, each sequence was analyzed with the ICE Software of Synthego (https://ice.synthego.com/#/). Analyses were performed using a control sequence of NT cells. The ICE score showed editing by nonhomologous end joining (NHEJ).

Proliferation

Cell proliferation was analyzed using CellTiter-Blue reagent. Briefly, 1000 hABSCs were seeded in 96-well plates. After 24 hours, cells were treated with LPS and/or Ti as explained above. On days 1, 3, and 7, CellTiter-Blue reagent was added to the cell culture for 4 hours and fluorescence was measured at 560 nm on an Infinite M200 Pro Microplate Reader. Long-term proliferation rates were measured as follows: 50 000 hABSCs were seeded in 12-well plates and treated with LPS and/or Ti. When cell cultures reached 90%-100% confluence, cells were harvested, counted, and re-plated at the same concentration.

Statistical analysis

The statistical analysis was performed using the GraphPad Prism software. All data are represented as mean (SD) of, at least, 3 independent experiments. Normal distribution of all data was analyzed using the Shapiro-Wilk test. Multiple comparisons of the data were performed using the one-way or two-way analysis of variance, followed by Tukey's post-test. *P* values ≤ .05 were considered statistically significant.

Results

LPS and Ti induce inflammasome activation on hABSCs

It is well documented that bacterial components and Ti promote NLRP3 inflammasome activation in different cell types of the immune system, such as macrophages and T cells.[17,18] To investigate whether bacterial components and metal ions could exert any effect on the induction of NLRP3 and AIM2 pathways in hABSCs, they were cultured in the presence of LPS and/or 20 µg/mL of Ti. This concentration falls within the physiological range of patients with peri-implantitis (20-30 µg/mL).[22] NLRP3 expression levels were then analyzed by estimating both mRNA and protein levels. A significant

increase in both NLRP3 mRNA (Figure 1A) and protein (Figure 1B, 1C) expression was observed in cells cultured with LPS. Moreover, when LPS is combined with Ti, this increase becomes more pronounced. Furthermore, a slight increase in NLRP3 expression was observed in hABSCs treated only with Ti. On the other hand, although it is known that the AIM2 inflammasome signaling pathway is only activated in the presence of dsDNA, surprisingly, when hABSCs were treated with LPS we could observe a strong increase in AIM2 at mRNA (Figure 1A) and protein levels (Figure 1B, 1C). However, we could also detect a significant decrease in both mRNA and protein levels in cells that had been treated with LPS and Ti compared to those that had only been treated with LPS.

AIM2 cannot be directly activated via LPS. Knowing that LPS causes an increase in the synthesis of mitochondrial DNA and its subsequent release into the cytoplasm,[23,24] we hypothesized that, when released, this DNA can be recognized by AIM2. To test this hypothesis, we measured the gene expression levels of COX-1 and ND-2 on mtDNA isolated from the cytosolic fraction of cultured cells. We used hABSCs treated with thapsigargin as a positive control since thapsigargin is known to increase mtDNA release to the cytosol.[25] We observed that, as we hypothesized, COX-1 and ND-2 expression levels are higher in LPS-treated hABSCs compared with hABSCs alone. The release of DNA from the mitochondria was not affected by the presence of Ti (Supplementary Figure S2).

Based on the differences that we observed in the expression levels of NLRP3 and AIM2 in the presence of bacterial components and/or Ti, we explored the possibility that these differences implied changes in the expression levels of the mediator caspase 1 (CASP1) and the proinflammatory molecule IL-1β. mRNA relative expression levels of both genes were quantified by qPCR and a significant increase in CASP1 and IL-1β expression was observed when hABSCs were treated with LPS. Again, the combination of LPS and Ti led to a higher increase in the mRNA levels of both genes (Figure 1D). ELISA analysis showed a significant increase in IL-1β secretion in cells cultured with LPS and an even greater increase when we combined the bacterial components with Ti. Interestingly, we were also able to observe a slight increase in IL-1β levels in the supernatants of those cells that were treated with Ti alone (Figure 1E).

Generation of NLRP3 and AIM2 knockout hABSCs

In order to generate hABSCs in which NLRP3 or AIM2 expression has been abolished, the CRISPR/Cas9 system was used. For this, we used "all-in-one" LVs in which Cas9 and the gRNA are encoded in the same vector. First, 3 specific gRNAs targeting different loci of each gene were designed. In the case of NLRP3, gRNA-1 targets the start codon (ATG) in exon 1, gRNA-2 targets another internal region of exon 1, and, finally, gRNA-3 targets an internal region of exon 3 (Figure 2A, left panel). In the case of AIM2, gRNAs 1, 2, and 3 target, respectively, specific regions of exons 2, 3, and 4 of this gene (Figure 2A, right panel). hABSCs were transduced with lentiviral particles containing the different vectors, hABSCs that had integrated the vector were selected with puromycin and, finally, genomic DNA was extracted and sequenced to check the knockout efficiency obtained (Figure 2B). The highest NLRP3 editing efficiency was 50%-60% using gRNA-3 and for AIM2, the highest efficiency, around 80%, was also achieved with gRNA-3 (Figure 2C). Therefore, gRNA-3 from both genes was selected for further analyses. In the case of both NLRP3 and AIM2 genes, the DNA cutting by Cas9 and its subsequent repair by nonhomologous end joining generated the insertion of a single nucleotide at the cutting site. Specifically, the inserted nucleotide was a thymine (Figure 2D). No significant off-targets were found (Supplementary Table S3).

The introduction of a single nucleotide should disrupt the reading frame of mRNA, leading to the synthesis of a truncated, larger, or nonfunctional protein. Frameshift insertions did not affect the levels of NLRP3 mRNA present in NLRP3-KO hABSCs, however, AIM2-KO hABSCs showed significantly lower mRNA levels of AIM2 compared to NT or transduced with the control vector (CTRL) hABSCs (Figure 3A). Regarding protein levels, a significant decrease in the expression of both genes was observed (Figure 3B, 3C).

NLRP3 and AIM2 expression is not affected by LPS and Ti treatment in knockout hABSCs

As shown above, LPS treatment significantly increases the mRNA and protein levels of NLRP3 and AIM2 in hABSCs. The combination of LPS and Ti leads to an even greater increase of NLRP3 expression and a decrease in AIM2. Based on these results, we analyzed the effect of LPS and Ti treatments in knockout hABSCs compared to CTRL hABSCs. As occurs in NT hABSCs, NLRP3 expression was induced in CTRL hABSCs cultured in the presence of LPS and this induction was higher when cells were treated simultaneously with LPS and Ti. However, although only 50%-60% of edited hABSCs for NLRP3 have been achieved, this number of edited cells is sufficient to observe that the treatment with LPS, Ti, or both does not have any significant effect on the expression of NLRP3 (Figure 4A, 4C, top panels). Concerning AIM2, we could observe that, as expected, the effect of LPS and Ti treatment is the same in NT and CTRL hABSCs. In the case of AIM2-KO hABSCs, according to the high percentage of gene editing, the addition of LPS and/or Ti to the cell cultures, does not have any significant effect on AIM2 expression levels (Figure 4B, 4C, bottom panels).

IL-1β secretion is lower in knockout hABSCs after LPS and Ti treatment

Once the effect on NLRP3 and AIM2 expression was analyzed in KO hABSCs, we wanted to check if these differences also implied changes in the expression levels of CASP1 and the secretion of active IL-1β. Comparison between CASP1 mRNA levels on NT, CTRL, NLRP3-KO, and AIM2-KO hABSCs did not evidence any significant difference (Figure 5A); however, when CTRL and KO hABSCs were subjected to LPS and Ti treatment, a decrease in the level of CASP1 mRNA was observed in AIM2-KO hABSCs compared to CTRL hABSCs with all the treatments. These differences were higher and became significant in the case of NLRP3-KO hABSCs in comparison with CTRL hABSCs (Figure 5B).

Nontreated NT, CTRL, NLRP3-KO, and AIM2-KO hABSCs showed similar levels of active IL-1β secretion (Figure 5C) but, again, when CTRL and KO hABSCs were cultured in the presence of LPS and/or Ti, a decrease in the secretion levels of IL-1β was observed in AIM2-KO hABSCs in comparison with CTRL hABSCs. This decrease became significant in those AIM2-KO hABSCs treated with LPS in combination with Ti compared to the same treatment in CTRL hABSCs. In the case of NLRP3-KO hABSCs, as occurs with CASP1, the

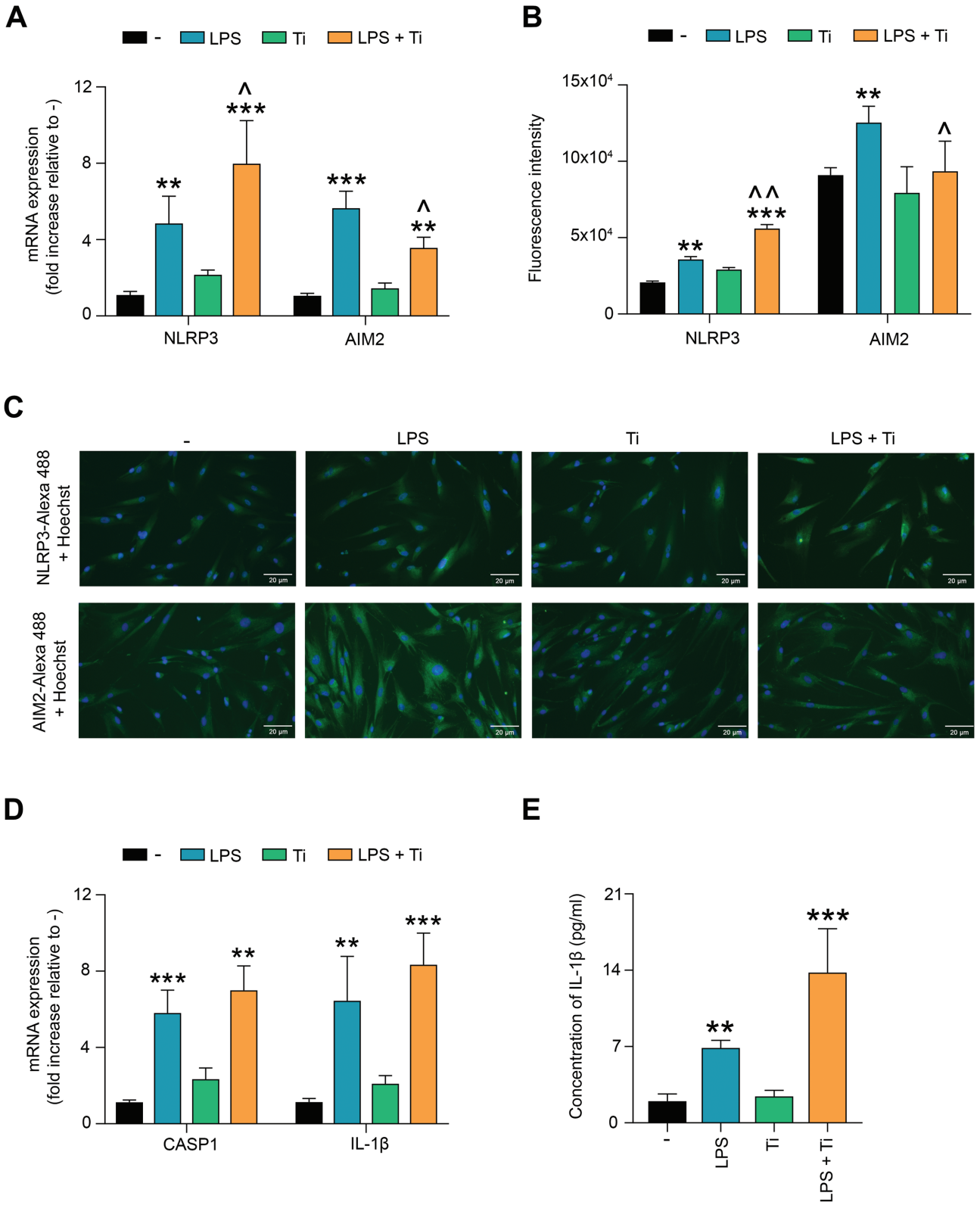


Figure 1. (A) hABSCs were treated with LPS, Ti, or LPS + Ti for 24 hours and NLRP3 and AIM2 expression was analyzed by RT-qPCR. (B, C) Nontreated (-) and treated hABSCs were fixed, permeabilized, and stained for anti-human NLRP3 or anti-human AIM2 antibodies. Quantification of NLPR3 and AIM2 fluorescence intensity is shown in (B) and one representative image of each individual staining is shown in (C). Hoechst dye was used to visualize the nuclei. Fluorescence intensity analysis of each experiment was performed using at least 50 individual cells of each condition. (D) RT-qPCR analysis of CASP1 and IL-1β expression in hABSCs treated with LPS, Ti, or both for 24 hours. (E) Nontreated, LPS, Ti, and LPS + Ti hABSCs were cultured for 48 hours and the levels of IL-1β were measured in supernatants by ELISA. Data are shown as mean (SD) of at least 3 independent experiments. $^{**}P < .01$; $^{***}P < .001$ versus (-); $^{\wedge}P < .05$; $^{\wedge\wedge}P < .01$ versus LPS.

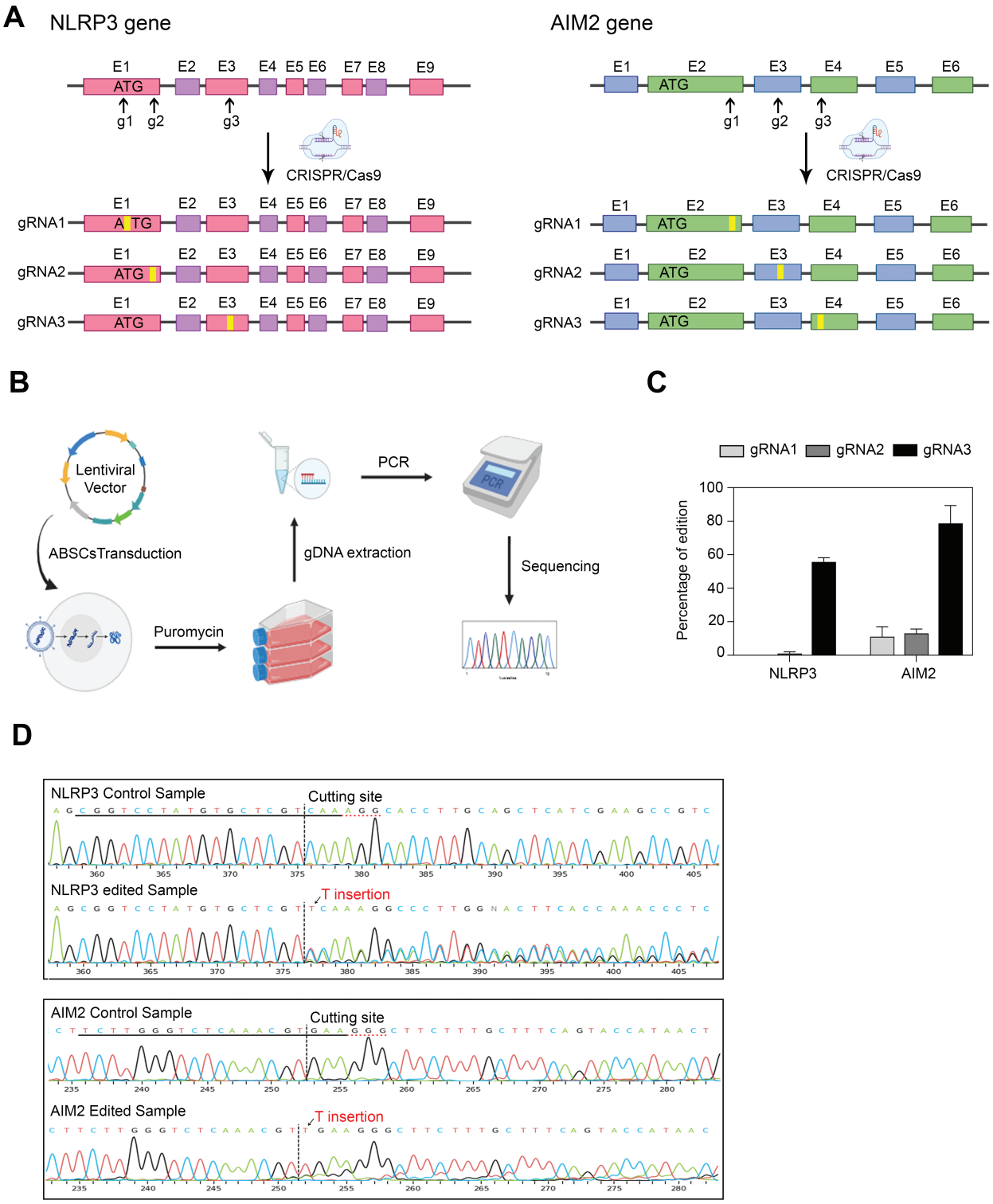


Figure 2. (A) Representative scheme of NLRP3 (left panel) and AIM2 (right panel) genes. Black arrows indicate the exon where the differently designed gRNAs are targeted. Yellow squares indicate the point where the mutations of NLRP3 and AIM2 genes will be generated once the Cas9 protein exerts its action. (B) Representative scheme of the transduction process using LVs and PCR analysis of genomic DNA to check gene editing efficiency. Image created with BioRender.com. (C) Editing efficiencies by CRISPR/Cas9 targeted at different exons in the human NRLP3 and AIM2 loci determined by the ICE algorithm. (D) Chromatogram showing edited and wild-type (control) sequences of NLRP3 (top graph) and AIM2 (bottom graph) genes in the region around the Cas9 cutting site. The horizontal black underlined region represents de gRNA sequence. The vertical black dotted line represents the actual cut site. DNA repair after the cut by Cas9 resulted in the insertion of a thymine just after the cutting site followed by mixed sequencing bases.

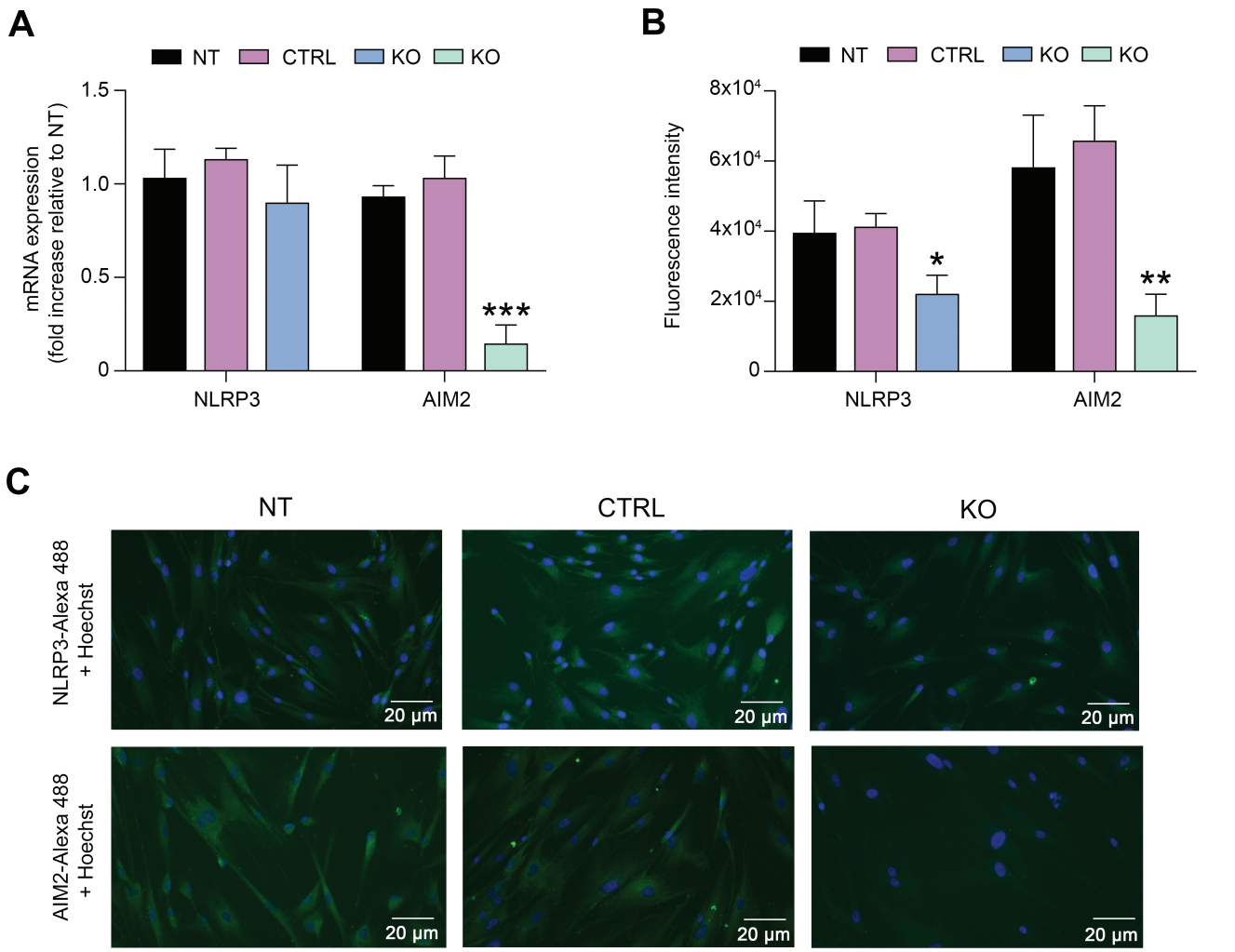


Figure 3. (A) hABSCs were transduced with LVs encoding a nonspecific gRNA (CTRL) or specific NLRP3 or AIM2 (KO) gRNAs. NLRP3 and AIM2 expression was assessed by RT-qPCR. (B) Quantification of NLRP3 and AIM2 fluorescence intensity in NT, CTRL, NLRP3, or AIM2 knockout hABSCs. The fluorescence intensity of each experiment was performed using at least 50 individual cells of each condition. (C) One representative image of each individual staining is shown. Hoechst dye was used to visualize the nuclei. Data are shown as mean of 3 independent experiments. *$P < .05$; **$P < .01$; ***$P < .001$ versus NT.

decrease in IL-1β secretion was higher and significant with all the treatments (Figure 5D).

LPS and Ti ions block hABSCs proliferation and induce the pyroptotic process

It is known that NLRP3 and AIM2 exhibit opposite effects on cell proliferation. While several studies have shown that the activation of NLRP3 promotes the proliferation of various cell types,[26,27] the activation of AIM2 leads to a decrease in the proliferative capacity of different cancer cell lines.[28,29] Thus, we analyzed the effect of NLRP3 and AIM2 knockout on hABSCs proliferation for 1 week. We could see that hABSCs transduced with the CTRL vector show similar rates of proliferation that NT cells, however, both NLRP3 and AIM2 KO cells exhibit a diminished proliferation capacity compared to unedited cells (Figure 6A). Then, we performed long-term proliferation analyses in edited and unedited cells that had been subjected to LPS and/or Ti treatments. In both NT and CTRL cells, the presence of LPS significantly decreases their proliferation capacity. This decrease was even greater when, in addition to LPS, hABSCs were treated with Ti. NT and CTRL hABSCs treated with Ti alone show similar rates of proliferation that nontreated cells (Figure 6B, 6C). Interestingly, in NLRP3 and AIM2 knockout hABSCs, the presence of LPS or LPS + Ti has a less pronounced effect on proliferation showing, especially those cells treated only with LPS, a proliferation capacity similar to untreated cells. (Figure 6D, 6E). Although KO cells with no treatment exhibit a lower proliferation ratio compared to unedited cells, under conditions in which they are cultured in the presence of LPS, the number of NLRP3 and AIM2 KO cells is significantly greater than the number of CTRL cells. This significant difference also occurs between CTRL and NLRP3-KO cells when they are cultured with LPS + Ti (Figure 6F).

We next investigated whether this decrease in cell survival when hABSCs are cultured in the presence of LPS or LPS + Ti was due to pyroptosis. By RT-qPCR we could observe that, in fact, mRNA levels of GSDMD increase in LPS-treated hABSCs compared with nontreated cells and this increase was greater and statistically significant in the presence of LPS and Ti (Supplementary Figure S3A). Comparison of GSDMD levels between nontreated edited and unedited hABSCs showed no significant differences (Supplementary

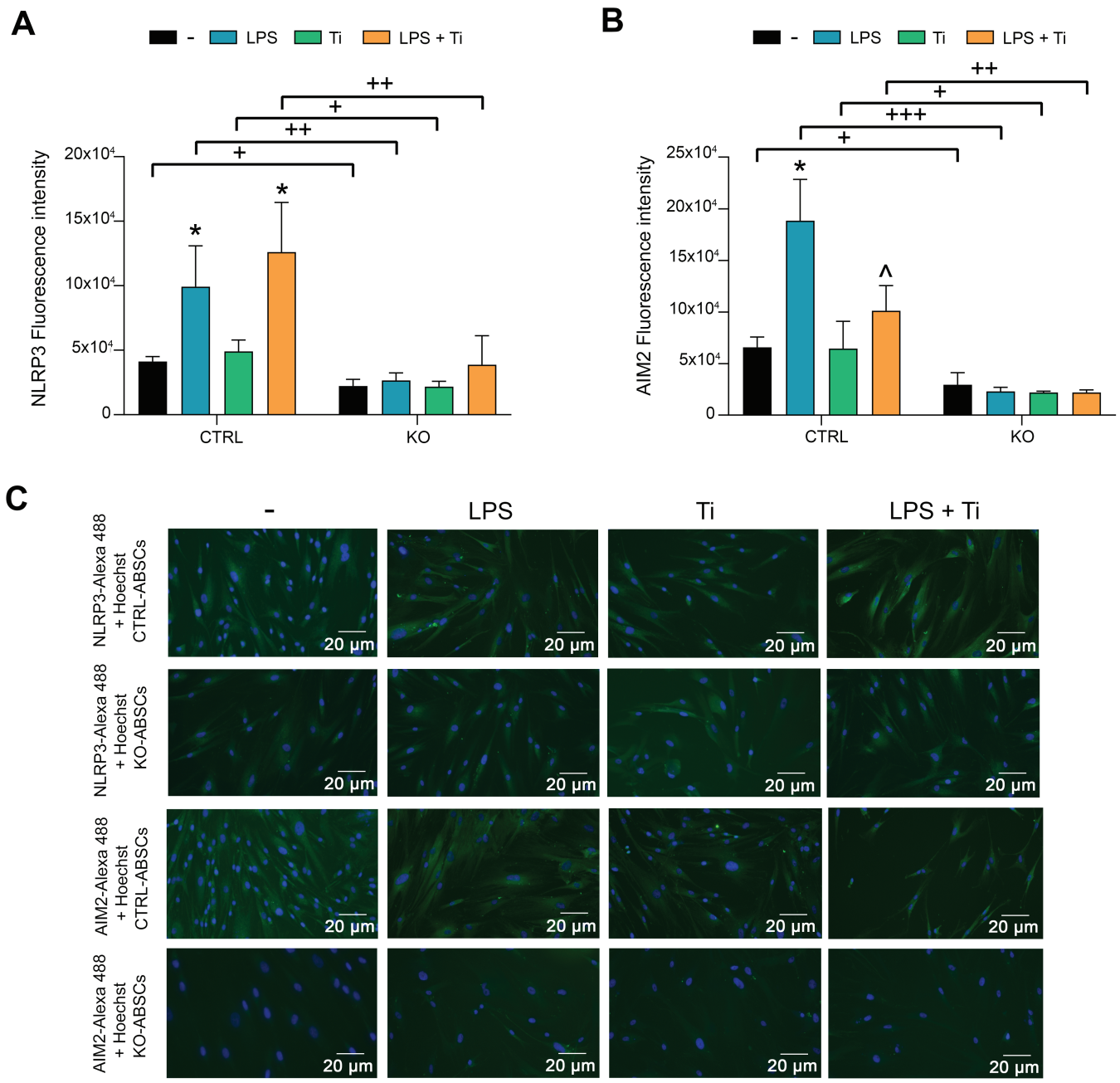


Figure 4. (A, B) CTRL, NLRP3-KO, and AIM2-KO transduced hABSCs were treated with LPS, Ti, or LPS + Ti. Quantification of NLRP3 (A) and AIM2 (B) fluorescence intensity. Fluorescence intensity analysis of each experiment was performed using at least 50 individual cells of each condition. (C) One representative image of each individual staining is shown. Hoechst dye was used to visualize the nuclei. Data are shown as mean of 3 independent experiments. *$P < .05$; **$P < .01$ versus (-); ^$P < .05$ versus LPS; +$P < .05$; ++$P < .01$; +++$P < .001$ CTRL versus KO among the treatments indicated by the square bracket.

Figure S3B); however, RT-qPCR analyses of GSDMD levels in CTRL hABSCs treated with LPS and/or Ti compared to NLRP3 and AIM2 knockout cells subjected to the same treatments showed a decrease in mRNA levels of GSDMD in both NLRP3 and AIM2 KO cells with all the treatments (Supplementary Figure S3C) suggesting a lower induction of the pyroptotic process in hABSCs in which the expression of NLRP3 or AIM2 has been partially decreased.

Taking into consideration the results obtained, we propose a model in which, in a periodontitis scenario, the presence of bacterial components (LPS) gives rise to the activation of NLRP3 and AIM2 inflammasomes, which in turn induces the activation of IL-1β gene and the subsequent secretion of this interleukin, as well as an increase in the levels of active GSDMD that will lead to the activation of the pyroptosis process giving rise to a decrease in hABSCs survival (Figure 7A). On the other hand, in a peri-implantitis scenario when, in addition to bacterial components, there is also metal (titanium), an increase in the activation of NLRP3 and a decrease in the activation of AIM2 inflammasome takes place. This increased activation of NLRP3 leads to an increased activation and secretion of IL-1β as well as to a greater entry of cells into pyroptosis compared to the scenario where only LPS is present (Figure 7B).

Discussion

Most of the in vitro studies that have been carried out on the inflammatory process triggered in patients who suffer

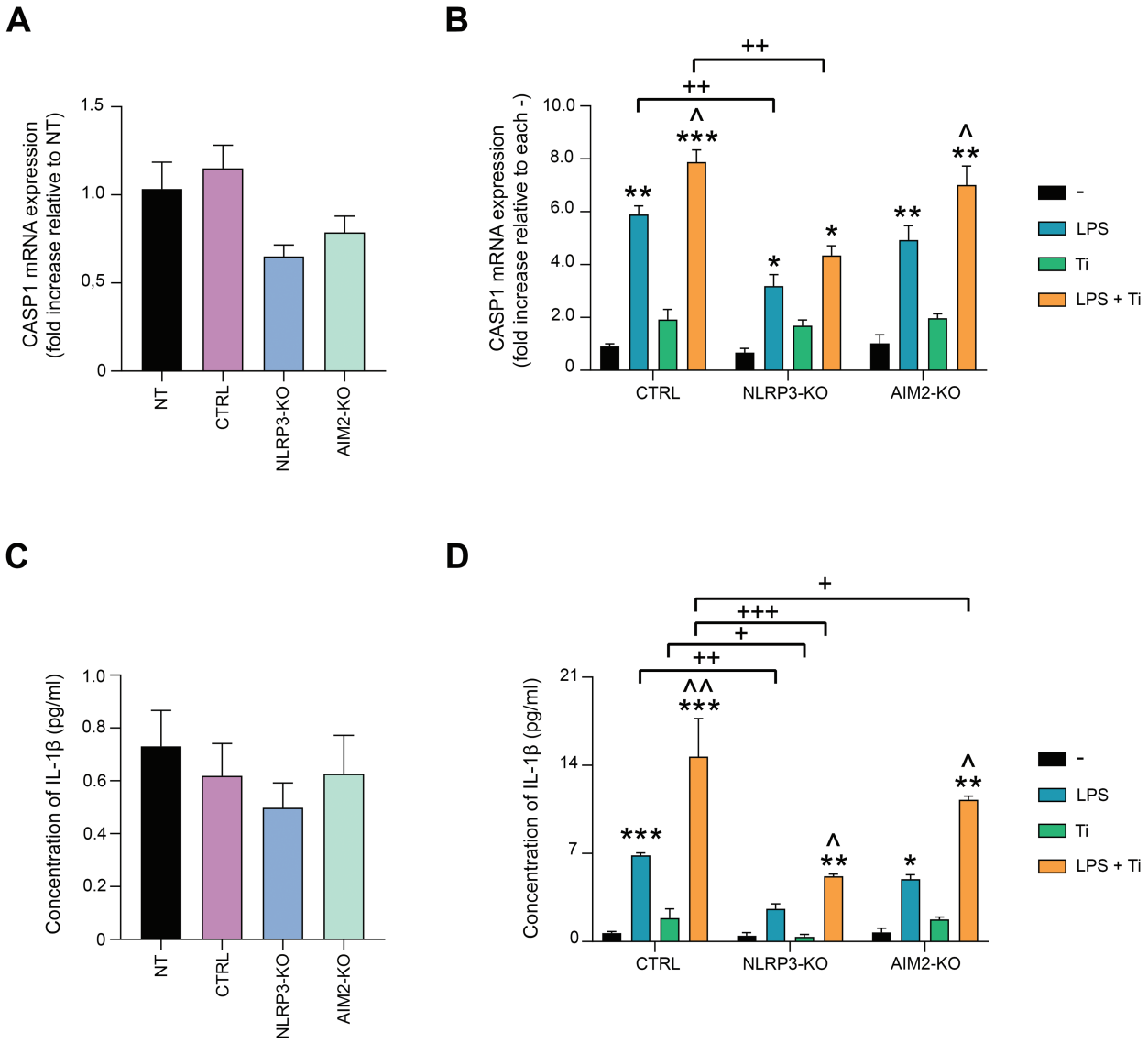


Figure 5. (A) RT-qPCR analysis of CASP1 in NT, CTRL, NLRP3-KO, and AIM2-KO transduced hABSCs. (B) CTRL, NLRP3-KO, and AIM2-KO transduced hABSCs were treated with LPS, Ti or LPS + Ti, and CASP1 mRNA expression was analyzed by RT-qPCR. (C) ELISA measurement of secreted IL-1β in supernatants of NT, CTRL, NLRP3-KO, and AIM2-KO transduced hABSCs. (D) CTRL, NLRP3-KO, and AIM2-KO transduced hABSCs were treated with LPS, Ti or LPS + Ti, and the levels of IL-1β were measured in supernatants by ELISA. Data are shown as mean (SD) of 3 independent experiments. *$P < .05$; **$P < .01$; ***$P < .001$ versus NT; ^$P < .05$; ^^$P < .01$ versus LPS; +$P < .05$; ++$P < .01$; +++$P < .001$ CTRL versus KO among the treatments indicated by the square bracket.

periodontitis or peri-implantitis have focused mainly on cells of the immune system.[17,30] However, it is essential to consider the effect produced by the presence of an inflammatory environment on those cells involved in the regulation of the immune response and in the regeneration of the bone tissue that is lost in both pathologies. In that sense, the aim of the present study was to analyze the inflammatory process, focused on NLRP3 and AIM2 inflammasomes pathways, in hABSCs that were cultured in the presence of bacterial components (LPS), Ti or the combination of both.

It is well known that LPS produces an increase in mRNA and protein levels of NLRP3, CASP1 and IL-1β on MSCs derived from bone marrow and umbilical cord.[15,31] Our results agree with these previous studies; surprisingly, we could also observe an induction of AIM2 that we think could probably be due to the presence of mtDNA in the cytoplasm of the cells.[12] As far we know, this is the first study in which AIM2 induction was analyzed on MSCs. The activation of AIM2 in response to LPS is also an interesting and novel result of the current study.

In order to better understand the molecular processes that take place in peri-implant disease, it is important to consider not only the bacterial component but also the presence of metal particles and ions released from the surface of the implant. In previous studies, it has been shown that Ti induce NLRP3 expression in macrophages and T-cell lines.[17,18] However, the secretion of active IL-1β has been proven only in THP-1 cells (a human monocytic cell line)[18] but not in macrophages isolated from peripheral blood[32] or T-cell lines.[17] As it has been shown in THP-1 cells, we have observed a slight increase in active IL-1β secretion in hABSCs in response to Ti alone and, according to the results obtained by Pettersson et al[32], this increase is significantly greater when LPS is combined with Ti ions. Thus, we show that MSCs not only respond to IL-1β[33]

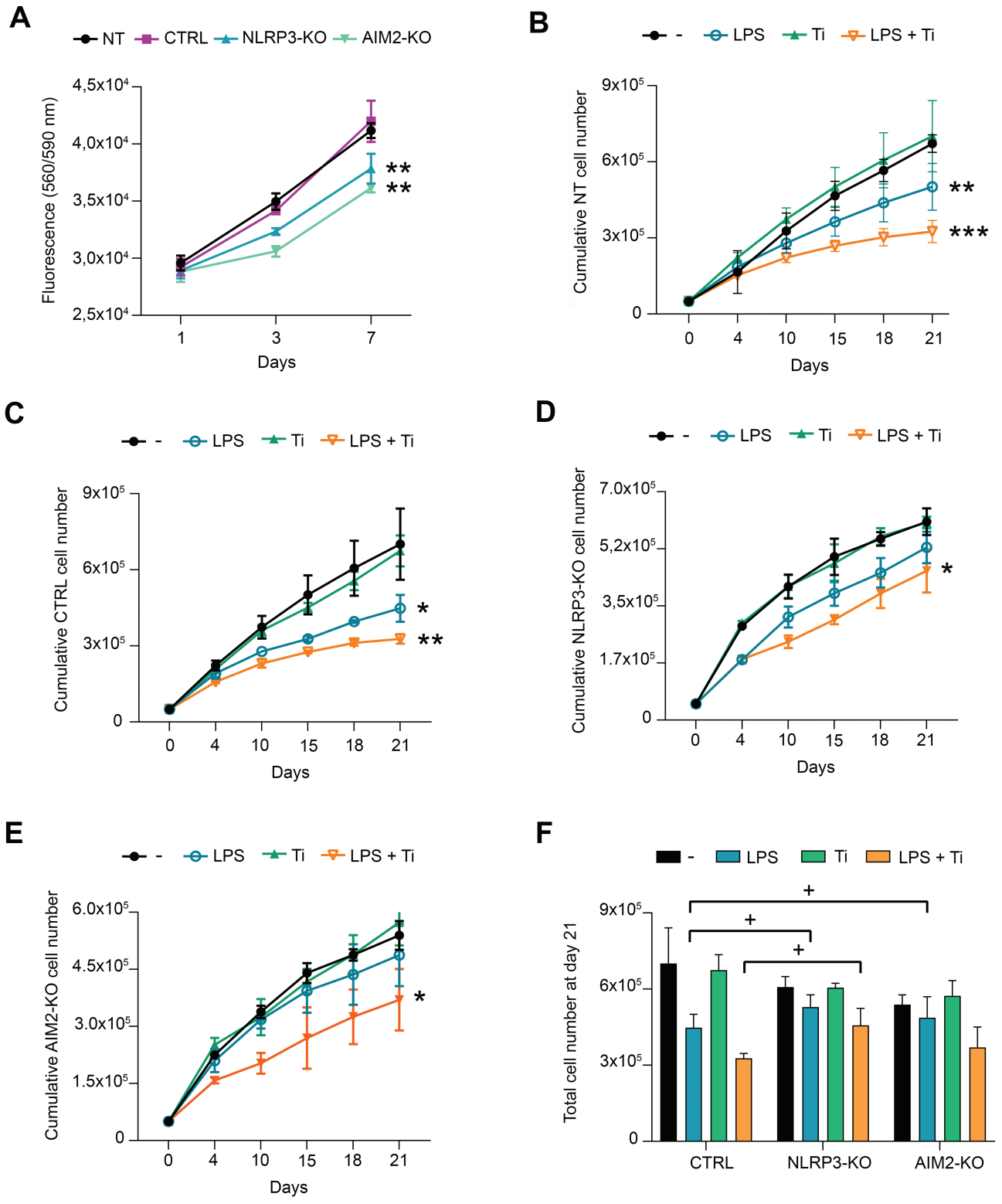


Figure 6. (A) NT (circle), CTRL (square), NLRP3-KO (triangle), and AIM2-KO (inverted triangle) hABSCs were plated in 96-well plates and after 1, 3, and 7 days CellTiter-Blue was added to the culture plates and fluorescence was measured at 560 nm. Grow curves of NT (B), CTRL (C), NLRP3-KO (D), and AIM2-KO (E) hABSCs treated with LPS (open circles), Ti (filled triangles), or LPS + Ti (open triangles) were generated by analyzing the cumulative cell numbers over time. Nontreated (filled circles) hABSCs were used as control. (F) Total number of untreated (-), LPS, Ti or LPS + Ti treated CTRL, NLRP3-KO, and AIM2-KO hABSCs at the endpoint of the experiment (day 21). Data are shown as mean (SD) of 3 independent experiments. *$P < .05$; **$P < .01$ versus (-); +$P < .05$ CTRL versus KO among the treatments indicated by the square bracket.

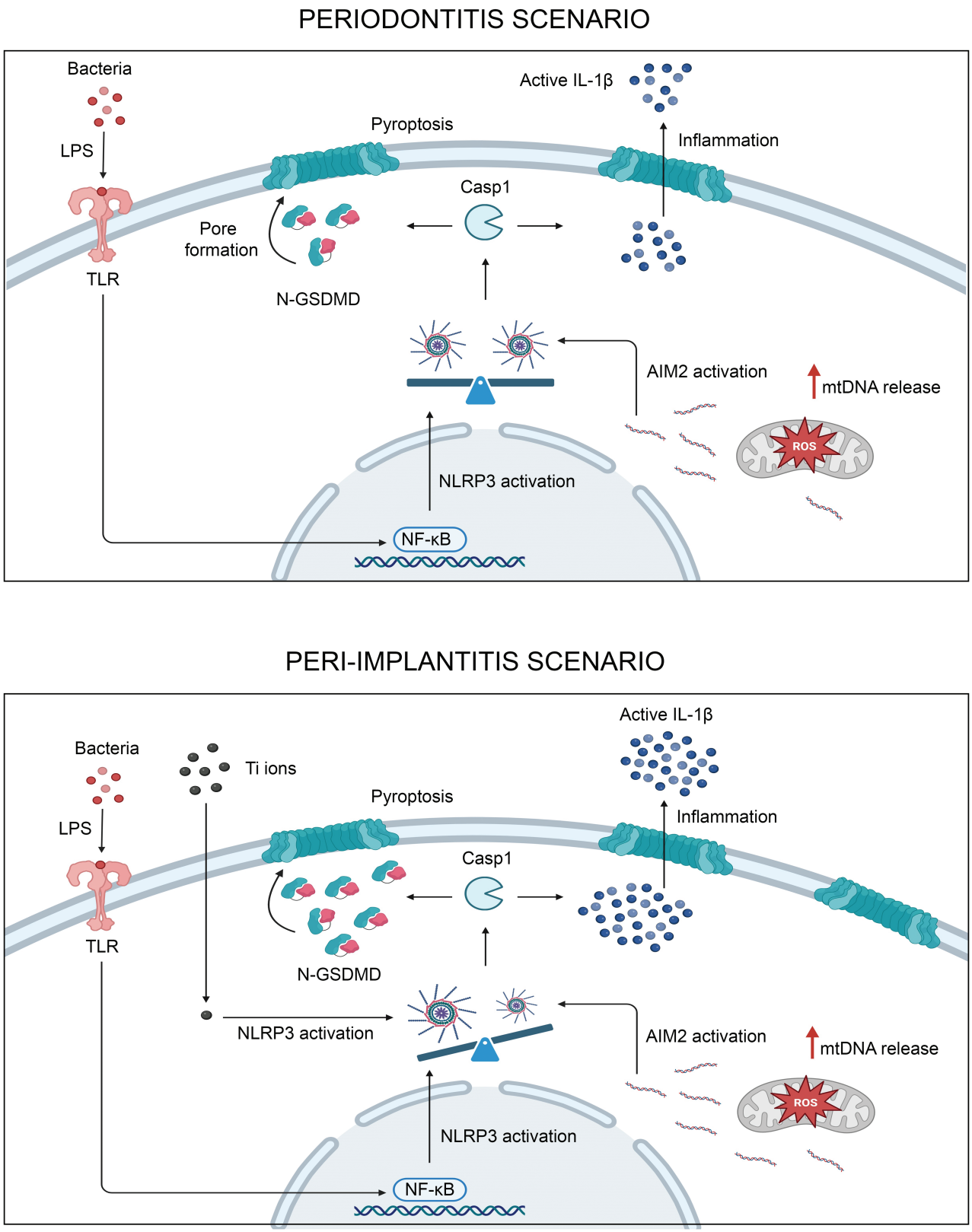


Figure 7. In a periodontitis scenario, bacterial components induce NLRP3 and AIM2 assembly and activation in hABSCs. This activation leads to an increase in the levels of secreted IL-1β proinflammatory cytokine and a conformational change of the GSDMD protein that leads to the formation of pores in the cell membrane, triggering the pyroptosis process. In a peri-implantitis scenario, the presence of metallic components leads to a greater activation of NLRP3 and a lower activation of AIM2 inflammasomes. This overactivation of NLRP3 inflammasome results in an increase in IL-1β secretion and in the formation of a greater number of pores in the cell membrane causing a higher cell death due to pyroptosis. Created with BioRender.com.

but are also capable of secreting and activating it themselves. This finding strongly suggests a synergistic activity of the metal ions and bacterial components in the induction of the inflammatory process on hABSCs. Importantly, we have also shown that AIM2 expression is significantly reduced in the presence of LPS and Ti ions compared with AIM2 expression in hABSCs cultured with LPS alone, pointing out that when metallic component is present, NLRP3 pathway takes prevalence over the AIM2 pathway. Probably, these 2 pathways are regulating each other, and, in this way, the homeostasis is restored via inflammatory response.

As we have observed in hABSCs, it is well documented that NLRP3 and AIM2 are upregulated in periapical lesions and gingival tissues from patients suffering from periodontitis; this activation leads to an increase in IL-1β secretion.[34,35] The expression of other inflammasomes such as NLRP2, NLRP8, and NLRP12 in peri-implantitis have been analyzed,[36] and recently, we have also shown that chronic inflammation in patients who suffer peri-implantitis can be explained in part by the action of IL-1β/CASP1 induced through NLRP3 and AIM2 inflammasome activation.[37] Little is known regarding the action of Ti in peri-implantitis; according to our results, Bressan et al[38] showed that Ti nanoparticles are present in peri-implantitis lesions and could trigger a more intense inflammatory response while Li et al suggested an induction of inflammasome activation in peri-implantitis due to the presence of metal ions and particles.[24]

On the other hand, it is important to consider the effect that the presence of bacterial and/or metallic components has on the survival of hABSCs. In this study, we observed a decrease in cell survival when hABSCs were cultured with LPS and an even greater decrease when LPS and Ti ions were combined. This effect could be explained, at least in part, due to the increase in the pyroptotic process that occurs in these cells when NLRP3 and AIM2 expression is induced. It is also important to highlight that, consistent with what D'Esposito et al[39] have observed using 3 different Ti disks, Ti alone do not affect the survival of hABSCs. This result suggests that tissue regeneration around an implant after its placement should not be affected by the release of Ti ions/particles from the implant surface, but, also according to our results, in a pathological scenario in combination with bacterial byproducts, the inflammatory process is activated more intensively because of the combination of titanium and bacteria.

Currently, many investigations are focused on analyzing the effect of suppressing NLRP3 expression using different inhibitors such as dopamine, CY-9, or anthracycline on various cell liness. These studies have shown how suppression of NLRP3 expression triggers a decrease in IL-1β activation and cell death due to pyroptosis in mouse[40,41] and human macrophages.[42] To our knowledge, there are no studies in which AIM2 expression has been suppressed to see its effect on the inflammatory process. In this report, we have generated loss-of-function mutants for both genes in hABSCs. First, we have successfully obtained approximately 60% NLRP3-edited hABSCs. We have not achieved a high editing percentage, and this could be because none of the gRNAs used were efficient enough. So, it would be necessary to design new gRNAs and use them to improve this editing efficiency. In the case of AIM2, we could obtain around 80% of edited hABSCs, which results in a strong decrease in AIM2 protein levels and mRNA levels. The action of Cas9 in AIM2-KO hABSCs has produced an insertion of a single nucleotide, therefore, it would be expected that mRNA levels would not be affected. However, it is known that small mutations produced by Cas9 can generate the appearance of a premature stop codon or pronouncedly affect the stability of the synthesized mRNA[43,44] which in turn could explain this decrease in AIM2 mRNA levels. As we have previously shown, in an inflammatory environment (LPS + Ti), NLRP3 and AIM2 KO hABSCs are able to significantly reduce the secretion of active IL-1β compared to NT and CTRL hABSCs. Curiously, although the percentage of gene editing in NLRP3-KO hABSCs was lower than in AIM2 knockout hABSCs, the negative effect on IL-1β secretion was much greater. This may confirm our hypothesis that under inflammatory conditions, especially in the presence of metal, there is greater relevance of NLRP3 pathway. Our results agree with previously published studies in macrophages in which NLRP3 expression had been suppressed with different inhibitors.[41,42] Cell survival is negatively affected by knockdown of NLRP3 and AIM2 expression; however, in the presence of LPS and Ti, this negative effect is significantly less pronounced compared to NT and CTRL cells.

As we have described in the introduction, in an inflammatory environment, MSCs are essential due to their immunomodulatory and tissue regenerative capacities. In fact, MSCs are used to treat different inflammatory pathologies including periodontal and peri-implant diseases.[45-47] It is fundamental to consider the effect produced by the induction of NLPRP3 and AIM2 on MSCs because it could affect their therapeutic abilities. In that sense, it is known that an augmented NLRP3 expression leads to a decrease in the osteogenic capacity of MSCs derived from the human umbilical cord.[15] Additionally, in a recent publication, it has been shown that the inhibition of NLRP3 expression gives rise to an increase in the immunomodulatory capacity of MSCs spheroids[48] although other authors have observed opposite results analyzing the immunomodulatory capacity of umbilical cord MSCs.[49] In a MSC therapy context, it is also important to take cell survival into account because the immunoregulatory and tissue regenerative abilities of hABSCs could be affected if the cells begin to die. In that sense, we have obtained interesting results since, in the presence of an inflammatory environment, it could be better to use hABSCs knockout for NLRP3 and AIM2 since they have a better survival capacity compared to NT and CTRL cells. Our results agree with the study carried out by Pham et al[48], in which they observed that the inhibition of NLPRP3 expression increases the survival of MSCs spheroids. Future studies are needed to analyze in depth whether the suppression of NLRP3 or AIM2 expression on MSCs could improve their therapeutic efficiency.

Certain in vivo investigations employing NLRP3 or AIM2 KO mice have demonstrated a diminished inflammatory response, characterized by reduced secretion of IL-1β and decreased mobilization of macrophages and neutrophils to the injury site.[50-53] This finding aligns with *our* in vitro results. However, surprisingly, divergent results have been reported in other studies utilizing KO mice for both genes, indicating an augmented inflammatory response.[54-56] Specifically, in ligature-induced periodontitis models, fewer osteoclast precursors, reduced alveolar bone loss, and attenuated inflammatory response have been observed in NLRP3 KO mice compared to wild-type mice.[50,57] Conversely, in the case of AIM2, no disparities in alveolar bone loss have been observed between

wild-type and AIM2 KO mice.[58] Further in vivo studies are warranted to elucidate the impact of suppressing NLRP3 and AIM2 expression on the progression of periodontal and peri-implant diseases.

Conclusion

In summary, our data suggest that, on the one hand, the progression of the inflammatory process in peri-implantitis conditions could be more acute due to the combined action of organic and inorganic components that increase the activation of the NLRP3 inflammasome, leading to decreased MSC survival and, on the other hand, the reduction of NLRP3 and AIM2 expression leads to a lower inflammatory response in the presence of bacterial and/or metallic components, as well as a better survival capacity of hABSCs, which could be beneficial for their use in therapy.

Author contributions

Ana Belén Carrillo-Gálvez: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing and final approval of manuscript. Federico Zurita: conception and design, collection and/or assembly of data, data analysis and interpretation, financial support, manuscript writing and final approval of manuscript. José Antonio Guerra-Valverde, Araceli Aguilar-González, Darío Abril-García: collection and/or assembly of data, data analysis and interpretation, and final approval of manuscript. Miguel Padial-Molina: povision of study material or patients, data analysis and interpretation, and final approval of manuscript. Allinson Olaechea, Natividad Martín-Morales: collection and/or assembly of data and final approval of manuscript. Francisco Martín: data analysis and interpretation, financial support and final approval of manuscript. Francisco O'Valle: data analysis and interpretation and final approval of manuscript. Pablo Galindo-Moreno: conception and design, provision of study material or patients, financial support, manuscript writing and final approval of manuscript.

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Conflicts of interest

The authors declare no potential conflicts of interest.

Data availability

Data supporting the findings presented in this study are available from authors upon reasonable request.

Supplementary material

Supplementary material is available at *Stem Cells Translational Medicine* online.

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