DOWN REGULATION OF IL-1 β SECRETION BY TGF- β 1 IN MACROPHAGES INFECTED WITH DENGUE VIRUS

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Abstract

Several pathogenic mechanisms have been linked to the severity of dengue virus infection, like viral cytotoxicity, underlying host genetics and comorbidities such as diabetes and dyslipidemia. It has been observed that patients with severe manifestations develop an uncontrolled immune response, with an increase in pro-inflammatory cytokines such as TNF, IL-1 β , IL-8, IL-6 and chemokines that damage the human microvascular endothelium, and also in anti-inflammatory cytokines IL-4, IL-10 and TGF- β 1. The role of TGF- β 1 on dengue is not clear; few studies have been published, and most of them from patient sera data, with both protective and pathological roles have been described. The aim of this study was to evaluate the ability of TGF- β 1 to regulate the secretion of IL-1 β in macrophages infected by DENV using THP-1 cells treated with recombinant TGF- β 1 before or after DENV infection. By RT-PCR we did not observe a difference in IL-1 β expression between infected cells pretreated with TGF- β 1 and those that were not. However, secretion of IL-1 β was reduced only in cells stimulated with TGF- β 1 before infection, and not in those treated 2 hours post-infection. TGF- β 1 receptor blockage with SB505124 inhibitor, prior to the addition of TGF- β 1 and infection, abrogated the inhibitory effect of TGF- β 1. Our results suggest that DENV could regulate the function of TGF- β 1 on macrophages. This negative regulation of the TGF- β 1 pathway could be used by DENV to evade the immune response and could contribute to the immunopathology.

Introduction

Despite health prevention campaigns and the recent application of a licensed vaccine, mortality and the disease burden metrics, such as years of life lost (YLLs), associated to Dengue increased by 65.5% and 32% respectively, from 1998 to 2017 (Collaborators 2018).

Humans infected by dengue virus (DENV) can present a wide array of manifestations but the great majority will not develop a serious condition, 20 to 25% will be symptomatic and only a small proportion will develop clinically relevant complications, including secondary symptoms associated to capillary leakage and organ involvement that will require hospitalization (Martinez, Garza et al. 2019,

Wilder-Smith, Ooi et al. 2019). Patients with dengue have a general increase in cytokine titers and severity has been associated to at least 25 different serum molecules, mainly TNF, IFN γ , IP-10, RANTES, chemokines, IL-1 β , IL-4, IL-6, IL-7, IL-8, IL-10 and TGF- β 1 (Pandey, Jain et al. 2015, Soo, Khalid et al. 2017, Srikiatkachorn, Mathew et al. 2017, Patra, Mallik et al. 2019). It has been proposed that the disease is primarily immune mediated, because the dysregulation of TNF and IL-6, among others, leads to vascular alterations, plasma leakage, thrombocytopenia and organ damage (St John and Rathore 2019).

IL-1 β is a prototype of inflammatory cytokine that has been associated to diverse pathologies, usually in combination with other cytokines such as TNF or IL-6. The role of IL-1 β in dengue pathogenesis is not completely understood. Studies in humans have shown contradictory results, some associated with an increase in the development of hemorrhagic symptoms (or severity of dengue), while others do not find a relationship at all (Chaturvedi, Nagar et al. 2006, Bozza, Cruz et al. 2008).

As in the case of IL-1 β , the role of TGF- β 1 on dengue pathogenesis is not clear. Some studies in patients associated an increase in TGF- β 1 with severity because levels were reduced in controls or in patients with dengue fever compared to dengue hemorrhagic fever; this difference seems to be more important particularly the day before the drop in temperature (Laur, Murgue et al. 1998, Agarwal, Elbishbishi et al. 1999, Patra, Mallik et al. 2019). However, polymorphism studies in dengue patients associated the genotypes of high TGF- β 1 producers with controls (Perez, Sierra et al. 2010, Yeo, Azhar et al. 2014). In addition, Tillu et al found a greater number of platelets in those patients with higher concentration of Tregs and TGF- β 1 (Tillu, Tripathy et al. 2016), suggesting a protective role for TGF- β 1.

In vitro, different studies have observed a relationship between TGF- β and IL-1 β . It has been observed that Smad6/7 proteins produced in response to TGF- β signaling induce a negative regulation of IL-1R/TLR signaling (Lee, Kim et al. 2010). Also, TGF- β can inhibit the production of IL-1 β in response to LPS through downregulation of CD14 (Imai, Takeshita et al. 2000). However, the relationship between these cytokines in the context of clinically expressed Dengue infection is still unknown. Therefore, we evaluated the role of TGF- β 1 in the regulation of IL-1 β production by macrophages infected with DENV.

Materials and Methods

Virus, cell culture, differentiation and infection of THP-1 cells

Dengue Virus serotype 2 New Guinea C was kindly donated by Dr. Humberto Lanz Mendoza, stock aliquots of 20 μ l were stored at -70°C, and diluted in Dubelcco's-PBS just before infection (SIGMA-Aldrich). THP-1 cells (ATCC Tib 202) were grown in Advance-RPMI 1640, supplemented with 3% Fetal Bovine Serum, (complete medium; all from GIBCO-Thermo Scientific) at 37°C and 5% CO₂. For differentiation into macrophages, cells were treated with 10 nM PMA (SIGMA-Aldrich) for 72 hours before infection or stimulation. Cells were differentiated in 24 well cell culture plates (Corning), 2x10⁵ cell/well, with sterile round glass coverslips for microscopy or ELISA quantification, or in 25 cm² flasks (Corning) at 4x10⁶ in 10 mL for RT-PCR assays. For infection, cells were washed with PBS, Dengue virus was added at a MOI=1 in a minimum volume of Dubelcco's PBS with CaCl₂ and antibiotics (SIGMA-Aldrich). After 2 hours, cells were washed with PBS and were further incubated for 24 hours in fresh complete medium. Infection was confirmed by fluorescent microscopy using anti-Dengue 1:20 (ab155042, AbCAM), anti-mouse Alexa 488 1:200 (Jackson Immunology), or by RT-PCR for prM/M-C region.

81 Effect of TGF- β 1 prior to infection with DENV on IL-1 β expression and secretion

82 For assays of effects of TGF- β 1 on the expression and secretion of IL-1 β , differentiated cells were
83 washed with PBS and fasted for 2 hours in RPMI-1640 (Gibco, Thermo Scientific) without serum, then
84 20 ng/mL of recombinant TGF- β 1 (PepProtech Inc.) was added and incubated for an additional 3 hours.
85 Cells were washed in PBS and infection proceeded as described.

86 Effect of TGF- β 1 post infection with DENV over IL-1 β expression and secretion

87 For the post-infection assay, fasted cells were infected with DENV in PBS for 2 hours as described,
88 and then complete medium was added with 20 ng/mL of TGF- β 1 (PepProtech Inc.). Cells were further
89 incubated for an additional 24 hours.

90 Effect of SB 505124 on IL-1 β secretion

91 Differentiated THP-1 cells were washed with PBS, fed with RPMI-1640 without serum, containing 1
92 mM of SB505124 inhibitor (TOCRIS Biosciences) and incubated for 2 hours. Then, 20 ng/mL of TGF-
93 β 1 was added, incubated for 3 hours before infection as described previously.

94 Stimulation of THP-1 with LPS

95 LPS was used to induce an inflammatory profile on THP-1 differentiated with PMA. The cells were
96 processed as for DENV infection, but in the final feeding with fresh complete medium, 10 ng/mL of
97 LPS from *E. coli* O11:B4 was added (Sigma-Aldrich).

98 Quantification of secreted IL-1 β

99 In all assays, 24 hours after infection, the supernatant was withdrawn, centrifuged at 575g for 10 min
100 (Spectrafuge, Labnet), aliquoted and stored at -20°C until used. In order to measure secreted IL-1 β we
101 used the commercial Human IL-1 β ELISA MAX kit (Biolegend), according to the manufacturer's
102 instruction.

103 Total RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

104 Total RNA was isolated from THP-1 cell lysate in Tri-Reagent (SIGMA-Aldrich) using a standard
105 organic solvents precipitation protocol according to the manufacturer's instruction.
106 Concentration/quality of total RNA was measured at 280/260 nm in a microvolume detection Take3
107 plate in an Epoch microplate spectrophotometer (Biotek), and agarose gel electrophoresis.
108 Complementary DNA (cDNA) was then synthesized from 1 μ g of total RNA using the Revert Aid First
109 Strand cDNA Synthesis Kit (Thermo Scientific), employing random hexamer primers. PCR was
110 performed using PCR Master Mix (2x) (Thermo Scientific) in a 3PRIME Thermal Cycler (TECHNE,
111 Bibby Scientific), according to manufacturer's instructions. Reaction conditions were, 35 cycles of
112 denaturalization at 95°C for 30 sec, annealing at specific temperature for each pair of primer, 60 sec
113 and extension 72°C for 30 sec. Specific primers and annealing temperature in °C for IL-1 β (57°C)
114 forward 5'GTCATTGCTCCACATTCT3', reverse 5'ACTTCTTGCCCCCTTTGAAT3';(Bozza,
115 Cruz et al. 2008); β actin (57-60°C) forward 5' AGGATCTTCATGAGGTAGT3', reverse
116 5'GCTCCGGCATGTGCAA3', TGF- β 1 (60°C) forward 5'GGTACCTGAACCCGTGTTGCT3',
117 reverse 5'TGTTGCTGTATTCTGGTACAGCTC3'. Products were analyzed by agarose gel
118 electrophoresis in a ChemiDoc XRS-Quantity One Image Analyzer (BioRad)

119 DENV RNA genome detected by PCR

120 A PCR assay was performed to detect infection and to amplify the RNA strand of DENV. Total RNA
121 of infected cells and synthesis of cDNA was conducted as described for IL-1 β . cDNA reaction was
122 adjusted to 500 ng/ μ l and used for PCR in Master Mix 2x (Thermo Scientific), using a 3PRIME
123 Thermal Cycler (TECHNE, Bibby Scientific). Reaction conditions were: 35 cycles denaturalization at
124 95°C for 20 sec, annealing at 60°C for 30 sec and extension at 72°C for 60 sec. The first PCR included
125 the following primers DENV ALL RV 5'CCCCATCTATTTCAGAATCCCTGC3' and DENV ALL F
126 5'CAATATGCTGAAACGAGAGAGAA3'. A nested PCR was performed with the amplicon from
127 first PCR product diluted 1:100 using the same reaction conditions for cycling, with the following pair
128 of oligonucleotides DENV ALL F 5'CAATATGCTGAAACGAGAGAGAA3 and DENV2RV
129 5'TGCTGTTGGTGGGATTGTTA 3'.

130 Statistical analysis

131 Descriptive statistics was used employing media and standard deviation (SD). Comparison between
132 experimental conditions was performed by using unpaired Students t-tests and $p < 0.05$ values were
133 considered as statistically significant. Graphs and statistic were performed using Prism v.8.1.0 software
134 (GraphPad).

135 Results

136 Treatment with TGF- β 1 reduces IL-1 β secretion only if it is applied before infection

137 In our experimental conditions, with a MOI=1 at 24 hours after infection, we observed 40% of infected
138 cells, confirmed by fluorescent microscopy and RT-PCR (Supplementary material). In these
139 conditions, infection with DENV induces the secretion of IL-1 β ($p < 0.005$). This stimulus was lower
140 than that induced by LPS. In order to determine if TGF- β 1 could have a regulatory effect on IL-1 β
141 secretion, we treated THP-1 differentiated cells with 20 ng/mL of recombinant TGF- β 1 for 3 hours
142 previous infections. This treatment resulted in a significant inhibition of the IL-1 β secretion ($p < 0.03$)
143 only in infected cells, but not in cells treated with LPS (Figure 1A). However, this effect was no longer
144 sustained if cells were first infected and then 2 hours later, treated with recombinant TGF- β 1 ($p = 0.46$),
145 (Figure 1B).

146 Inhibition of TGF- β 1 receptor abrogates the effect of TGF- β 1 over IL-1 β

147 In order to further validate this data, pretreatment of the THP1 with a selective inhibitor of TGF- β RI,
148 SB505124, was performed prior to exposure to 20 ng/mL of TGF- β 1; 3 hours later infection with
149 DENV proceeded as described above. This treatment abrogated the inhibitory effect of TGF- β 1 on
150 infected cells. In fact, after inhibitory pretreatment, we observed a higher increase of IL-1 β secretion
151 induced by DENV infection ($p < 0.04$), in comparison to non-inhibited cells. It is noteworthy that both
152 the uninfected cells and those treated with LPS also responded with an increase in the production of
153 IL-1 β (Figure 1C).

154 Effect of TGF- β 1 over IL-1 β secretion is not related with IL-1 β expression

155 IL-1 β is produced as a 31 kDa precursor that needs to be processed for its secretion. In order to
156 determine if the effect of TGF- β 1 is due to a repression of IL-1 β expression, we evaluated the IL-1 β
157 messenger using RT-PCR. Although infection and LPS treatment increased the expression of IL-1 β
158 RNA messenger, we did not observe any change when comparing the infected cells without treatment

159 with those who underwent treatment with TGF- β 1, suggesting that the effect of TGF- β 1 is major
160 associated to secretion.

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186 **Figures**

Figure 1. A. Percentage of fluorescent cells. Controls at 24 and 48 hpi, average 10-15% positive cells. DENV 24 and 48 hpi. 40% and 85% positive cells. B. IL-1β secretion in experimental groups adding TGF-β1 recombinant previous to infection. C. IL-1β secretion in cells adding TGF-β1 before viral infection. D. IL-1β secretion in different treatments using TGF-β1 receptor competitive inhibitor. Statistical significant differences were made using Student's T assay. P<0.05 *, p<0.01 **, p<0.005 ***.

Figure 2. Previous treatment with TGF-β1 does not modify mRNA IL-1β. A. mRNA IL-1β and β-actin in cells with TGF-β recombinant or PBS over 3 h before and after viral infection. B. Relative expression of IL-1β on TGF-β1 previous to infection was normalized to actin as pixel intensity per mm². C. Relative expression of IL-1β on TGF-β1 added post-infection was normalized to actin as pixel intensity per mm²

Discussion

The complexity of the disease caused by the infection of DENV is due to the fact that it depends on many factors, such as virus serotype and genotype, the mosquito proteins, previous infection with dengue or other arbovirus, host genetic polymorphism, co-morbidities such as obesity, cardiovascular disease or asthma (St John and Rathore 2019). However, it has been observed that the immune response of the host plays an important role in the pathogenesis of clinically expressed Dengue because it leads to an increase in inflammatory and anti-inflammatory cytokines (Chaturvedi, Nagar et al. 2006, Pang, Cardosa et al. 2007, St John and Rathore 2019). There is no clear proposal that explains how this cytokine imbalance can lead to a worsening of the disease, but one hypothesis is that different cytokines released during infection, such as TNF, IL-6 or IL-8, have a direct action on vasculature, leading to dysregulation of the endothelial cell barrier associated to increased vascular permeability, resulting in pleural effusion or ascites (Srikiatkhachorn, Mathew et al. 2017). The search of biomarkers that can

predict the severity of disease before symptoms appears has been going on for years. The 24 to 48 hour period before the drop in body temperature is especially important because vascular alterations, plasma leakage, increase hematocrit, lowering of platelets and organ commitment usually occurs 2-3 days after deffervescence (Yacoub and Wills 2014). So far it has not been possible to determine these biomarkers, not only because of patient variability, but because of methodological differences that include disease severity classifications, age of patients, the day on which the serum is obtained, etc (John, Lin et al. 2015).

At the end of 90's, several clinical studies associated the increased levels of TGF- β 1 with severity because levels were reduced in controls or in patients with dengue fever compared to dengue hemorrhagic fever; this difference seems to be more important particularly the day before the drop in temperature, and last for 9 day after fever onset (Letterio and Roberts 1998, Agarwal, Elbishbishi et al. 1999, Patra, Mallik et al. 2019). However, polymorphism studies comparing healthy or asymptomatic patients with those that developed severe dengue associated the genotypes of high TGF- β 1 producers with controls, suggesting a protective role for TGF- β 1 (Perez, Sierra et al. 2010, Yeo, Azhar et al. 2014). Interestingly Djamiatun et al. found that TGF- β 1 plasma levels always were lower than healthy children, even if there was an increase in patients with hemorrhagic manifestations (Djamiatun, Faradz et al. 2011). Otherwise *in vitro* study has shown that cells from a DENV serotype donor, produces higher TGF- β 1 when re-challenged with the same serotype, but not with a heterologous serotypes. As severe dengue has been associated to reinfection, this study could also suggest a protective role for TGF- β 1(Perez, Sierra et al. 2010, Sierra, Perez et al. 2010)

TGF- β 1 is a multifunctional cytokine which immunomodulatory profile changes depending on concentration, tissue location and context of action; for example on T cells, leading to the suppression of Th1, Th2 and CD8 activation and also favoring the generation of FoxP3/Tregs (Li and Flavell 2006, Chen and Ten Dijke 2016). Tillu et al. found elevated Tregs frequencies in patients with mild dengue disease, suggesting a protective role of these cells, and also noted a relationship between TGF- β 1 levels and greater number of platelets in those patients with higher concentration of Tregs (Tillu, Tripathy et al. 2016). On the other hand, effects of TGF- β 1 on macrophages can be either stimulating or inhibitory, depending on the presence of other cytokines and the state of maturation (Letterio and Roberts 1998, Travis and Sheppard 2014)

In order to establish if there is an association between TGF- β 1 and the macrophage response to DENV infection, we conducted an *in vitro* study using THP1 differentiated into macrophages. In our assay's condition, at 24 hours post-infection, 40% of cells were positive for DENV but we observed a significant increase in IL-1 β secretion. Other groups also have observed that macrophages infected with DENV, even in the absence of antibodies, secrete IL-1 β , up until 6 hours post-infection (Callaway, Smith et al. 2015). Here we observed a total reduction of IL-1 β secretion in infected cells that were treated 3 hours with TGF- β before infection, but not in cells stimulated with LPS. Concentration of IL-1 β released by macrophages stimulated by LPS was almost 5 times higher than those infected by DENV. This difference could be explained by the fact that LPS and DENV bind to different TLRs.

In our assay of pre-treated THP-1 cells with a selective inhibitor of TGF- β I receptor, SB-505124, that inhibit specific activation dependent of Smad2/3, the inhibitory effect of the TGF- β on the secretion of IL-1 β was abrogated. Additionally, once the infection started, we no longer observed the inhibitory effect of TGF- β 1 on IL-1 β secretion. For other part, we did not notice any change in IL-1 β RNA messenger using RT-PCR, suggesting that the mechanism of TGF- β 1 should be related to events after the expression of IL-1 β ; however, it is necessary to confirm this data by qPCR. Kwon et al., using siRNA assay shown that knock-down of Smad7, an inhibitory protein of TGF- β signaling, is associated

269 to a lesser infection. They also showed that infected U937-DC-Sign cells increased the expression of
270 Smad7 in response to DENV (Kwon, Heo et al. 2014). At this respect, we can assume that DENV
271 should manipulate the response to TGF- β 1 potentially by modification of the cellular pathways that is
272 why it should be important to evaluate the changes in expression and activation of Smad 2/3 and
273 Smad7.

274 IL-1 β is produced as a 34 kDa precursor that requires maturation by proteases, usually Caspase-1,
275 before secretion. IL-1 β is a leaderless cytosolic protein and the precise mechanism of release of this
276 cytokine is not completely understood, but it is known that it requires a non-canonical pathway
277 independent of Golgi. Several mechanisms have been proposed to explain the release of this cytokine
278 as pores associated to phosphatidylserine, autophagy vesicles or pyroptosis (Wu, Chen et al. 2013,
279 Brough, Pelegrin et al. 2017, New and Thomas 2019) .

280 We did not find previous evidence that TGF- β 1 has an effect on the inhibition of secretion of IL-1 β
281 that could be associated to the activation of caspase-1 and induction of pyroptosis. Some studies have
282 shown that DENV infected monocytes or macrophages died by pyroptosis after activation of caspase-
283 1 (Tan and Chu 2013, Wu, Chen et al. 2013). Another study has shown the role of caspase-4 on the
284 activation the maturation of IL-1 β in DENV infected cells (Cheung, Sze et al. 2018). Interestingly, the
285 secretion of IL-1 β was observed almost 90 hours before the activation of caspase-1 or cell death. It is
286 known that IL-1 β could be processed by other proteases and not necessarily due to its secretion leading
287 to cell death (Netea, van de Veerdonk et al. 2015). In contrast, here we observed elevated IL-1 β
288 secretion at 24 hours post-infection as others have reported (Callaway, Smith et al. 2015).

289 Autophagy has a dual role in the regulation of IL-1 β because it is required both for the maturation and
290 release, and also controls its release by its degradation before secretion. The autophagy vesicle required
291 for maturation and release of IL-1 β seems to be a non-classical vesicle. IL-1 β is associated to TRIM16
292 and after complex traffic to an intermediate membrane positive for LC3-II, and then a vesicle is formed
293 and associated to SEC22b. In order to reach the plasma membrane this vesicle undergoes a SNARE-
294 modified fusion (New and Thomas 2019). However, inhibitors of autophagy, or genetic defective
295 autophagy cells, result in an increase of IL-1 β secretion, but not of TNF or IL-6 on cells stimulated
296 with LPS. The autophagy vesicle formed in absence of an inflammasome-inducing signal is
297 independent of TRIM16 and co-localize with LAMP-1 and STX17, leading to transport and
298 degradation of IL-1 β by lysosomes (Harris 2011, Harris 2013).

299 During viral infection, the virus manipulates different cellular pathways, such as the response to IFNs
300 and autophagy. Several studies have shown that many autophagy induced pathways are activated by
301 DENV for their replication (Datan, Roy et al. 2016). Inhibition of ER stress signaling limits the ability
302 of DENV to induce autophagy and decrease the formation of mature virions (Datan, Roy et al. 2016).
303 It has been shown that proteins of the viral replication complex do not co-localize with autophagosome
304 membranes, suggesting that it may play another role, such as immune evasion response (Heaton and
305 Randall 2011). On the other hand, several studies have shown that TGF- β 1 increases autophagy,
306 mediated through the Smad and JKN pathways .

307 Our proposal is that in the absence of DENV infection, TGF- β 1 will induce the accumulation of IL-1 β
308 and inflammasome proteins in autophagic vesicles associated with STX17 and SNARE, directing the
309 complex to lysosomes. But when the dengue virus initiates the infection of macrophages, mediated by
310 TLR 3,4 and 7, the signal pathways lead to the accumulation of IL-1 β in vesicles associated to TRIM16
311 and SEC22b, eventually resulting in the secretion of IL-1 β . Evidently this proposal must be confirmed

by experimental data. Another mechanism that could explain why infected DENV macrophages do not respond to TGF- β 1 could be associated to signaling downstream pathways.

A limitation of our study is that we performed our assays in the absence of antibodies. It is known that the macrophage activation pathways are very different if the virus binds directly to the macrophages or is mediated by immunocomplexes and FcR γ . Callaway et al. have shown that Dengue-Antibody complexes can increase the secretion of IL-1 β , but only when the immunocomplex is generated by serum homologous to the serotype of the virus and not with heterologous sera (Callaway, Smith et al. 2015). This is also important because it is known that TGF- β 1 reduces the expression of FcR γ on the surface of cells.

In conclusion, this study supports the proposal that TGF- β 1 may have a limiting role in the development and severity of dengue disease, but only on those individuals who prior to infection have TGF- β 1 present in the cellular environment.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

RB, MJ and GJ contributed to conception, design and interpretation of results of the study. RB conducted the main laboratory work. SJ conducted the test for PCR detection of DENV and wrote material and methods section. All authors contributed to read, write and approval the final manuscript.

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Data Availability Statement

The dataset and raw data supporting the conclusion of this manuscript will be made available by authors, without undue reservation to any qualified researcher.

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436 Legends

437 Figure1. TGF- β 1 inhibits the secretion of IL-1 β in THP-1 infected with DENV. A) Differentiated THP-
 438 1 cells were pre-treated with 20 ng/mL of TGF- β 1 or with PBS for 3 hours before infection, following
 439 cells were infected with DENV (MOI=1) or treated with 10 ng/mL of LPS and incubated for additional
 440 24 hours; B) Differentiated THP-1 cells were first infected with DENV and 2 hours after treated with
 441 TGF- β 1, control cells were incubated with PBS or LPS; C) Differentiated THP-1 were firstly pre-
 442 incubated with 1 μ M SB505124 for 2 hours and then treated by TGF- β 1 for 3 hours before DENV
 443 infection. After 24 hours of infection, supernatant IL-1 β secretion was quantified by ELISA. Difference
 444 between treatments was evaluated by student t test, considering as $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.005^{***}$.

445 Figure2. TGF- β 1 treatment do not modify the expression of IL-1 β RNA messenger in THP-1 cells. A)
 446 Expression of IL-1 β RNA messenger or β -actin RNA messenger in cells incubated for 3 hours with 20
 447 ng/mL of TGF- β 1 or with PBS for 3 hours before DENV infection; B) Expression of IL-1 β RNA
 448 messenger or β -actin RNA messenger in cells infected for 2 hours with DENV following by stimulation
 449 for 3 hours by 20 ng/mL of TGF- β 1 or with PBS, total RNA was extracted after 24 hours of infection.
 450 Relative expression, was evaluated as intensity of pixels by area (INT/mm²) in IL-1 β bands and
 451 normalized with the corresponding β -actin band; C) graphical results of treatment by TGF- β 1 before
 452 DENV infection; D) graphical results of treatment by TGF-b1 after DENV infection.

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