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Rapamycin increases RSV RNA levels and survival of RSV-infected dendritic cell depending on T cell contact

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ABSTRACT

The macrolide rapamycin inhibits mTOR (mechanist target of rapamycin) function and has been broadly used to unveil the role of mTOR in immune responses. Inhibition of mTOR on dendritic cells (DC) can influence cellular immune response and the survival of DC. RSV is the most common cause of hospitalization in infants and is a high priority candidate to vaccine development. In this study we showed that rapamycin treatment on RSV-infected murine bone marrow-derived DC (BMDC) decreases the frequency of $CD8^+CD44^{high}$ T cells. However, inhibition of mTOR on RSV-infected BMDC did not modify the activation phenotype of these cells. RSV-RNA levels increase when infected BMDC were treated with rapamycin. Moreover, we observed that rapamycin diminishes apoptosis cell death of RSV-infected BMDC co-culture with T cells and this effect was abolished when the cells were co-cultured in a transwell system that prevents cell-to-cell contact or migration. Taken together, these data indicate that rapamycin treatment present a toxic effect on RSV-infected BMDC increasing RSV-RNA levels, affecting partially CD8 T cell differentiation and also increasing BMDC survival in a mechanism dependent on T cell contact.

1. Introduction

Rapamycin, the established inhibitor of mTOR (mechanist target of rapamycin), was discovered in soil samples from Easter Island (Rapa Nui) in the 1970s and found to have antifungal activity (Li et al., 2014). mTOR is a serine-threonine protein kinase, which plays a central role in regulating cell growth, metabolism and survival (Wullschleger et al., 2006). Since 1999, rapamycin is approved for acute renal allograft rejection. In 2007 and 2009 FDA approved two derivate of rapamycin, temsirolimus and everolimus, respectively for advanced renal cancer carcinoma treatment. Nowadays, it is known that pharmacological inhibition of mTOR by rapamycin has a wide range of clinical effects and future clinical applications (Hasty, 2010).

Dendritic cells (DC) are important antigen presenting cells that recognize, process and present antigen to T cells and regulate the immune response (Banchereau et al., 2000; Condon et al., 2011). These cells are essential for initiation T cell immunity against virus (Banchereau and Steinman, 1998). In result to rapamycin treatment a lot have been learned about the role of mTOR on DC. mTORC inhibition hampers murine bone marrow derived DC maturation (Hackstein et al., 2003) and endocytosis (Hackstein et al., 2002) impairing T cell differentiation (Turnquist et al., 2010). In contrast, inhibition of mTOR on DC during activation was related to the increase in the lifespan of these cells, the extension of co-stimulatory molecules expression and the induction of an effective and strong specific $CD8^+$ T cells response (Amiel et al., 2012).

Human Respiratory syncytial virus (RSV), an omnipresent virus, is the main cause of lower respiratory tract infection in newborn babies and very young children (Nair et al., 2010; Hall et al., 2009). Hospitalization numbers due to RSV infection in children are increasing each year and global annual death of 200,000 children is estimated in consequence of infection (Nair et al., 2010). Palivizumab, indicated to prevent severe infection in high-risk children, is a very expensive treatment (F. the A.A. of Pediatrics, 2009). Currently, there is no efficient RSV vaccine available, although there are promising approaches being tested in mouse model (Bueno et al., 2008; Anderson et al., 2013) and in clinical trials (Green et al., 2015a; Green et al., 2015b). RSV infection can impair murine (González et al., 2008; Ptaschinski et al., 2015) and human (Gupta et al., 2013) DC function preventing specific T cells activation. In addition, RSV infection induced limited plasmacytoid DC responses in mice (Cormier et al., 2014) and in human (Weng et al., 2014).

We recently demonstrated that RSV-infected infants present elevated mTOR expression comparing to uninfected ones (de Souza et al., 2016). In addition, rapamycin treatment on T cells enhances the frequency of mouse RSV-specific CD8 T cells memory precursors (de Souza et al., 2016). However, so far there is no information about mTOR inhibition on DC during RSV infection. Therefore, the main

Abbreviations: RSV, respiratory syncytial virus; BMDC, bone marrow derived dendritic cells; DC, dendritic cells; mTOR, mechanist target of rapamycin.

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objective of this study was to investigate the role of rapamycin treatment on DC during RSV infection.

2. Materials and methods

2.1. Mice

Female C57BL/6 mice ranging from 8 to 12 weeks old were purchased from Centro de Modelos Biológicos e Experimentais (Cembe) - PUCRS. Mice were housed at the animal facility of Cembe with water and food ad libitum. All animal procedures were performed in accordance with protocols approved by the University ethics Committee (CEUA Protocol number 14/00391).

2.2. Virus

RSV A strain (line A2) was provided by Fernando Polack, Fundación Infant, Argentina. The virus was grown in Vero cells. Viral plaque forming units (PFU) were identified using an RSV F protein-specific antibody (Millipore, Billerica, MA).

2.3. Murine bone marrow derived dendritic cells cultures

Murine bone marrow derived dendritic cells (BMDC) were grown from bone marrow in AIM-V medium (Gibco, Carlsbad, CA) with GM-CSF and IL-4 (Peprotech, Ribeirao Preto, Brazil), as previously described (Inaba et al., 1992).

2.4. Purification of T cells

Murine T cells were purified from spleen and lymph nodes (inguinal, axillary and brachial) using Pan T cells magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany (order number 130-095-130)), following the manufacturer's instructions.

2.5. Culture of T cells and RSV infected BMDCs

After 6 days of differentiation, 1.5×10^4 BMDCs were treated with 20 ng/mL of rapamycin for 1 h. Cells were washed and infected with 10^2 PFU of RSV for 1 h in RPMI medium without fetal bovine serum (FBS) and then washed to remove the virus. Purified T cells were seeded at concentration of 5.0×10^4/well in 96-well plate in co-culture with BMDC. Cells were co-cultured in RPMI with 5% FBS for 96 h at 37 °C at atmosphere containing 5% CO_2. For some experiments, cells were cultured in a 6.5 mm Transwell® system (CORNING, New York, USA (product number 3470)) with 0.4 μm pore membrane insert to avoid contact between them.

2.6. Flow cytometry

Cells were incubated with FcBlock (supernatant of 2.4G2 cells with 5% of human serum) for 20 min. T cells were stained with anti-CD8a (clone 53,67) and anti-CD44 (clone IM7) (BD Biosciences®) in dark for 30 min. BMDCs were stained with anti-CD11c (clone HC3), anti-CD86 (clone Rit-GL1) and anti-MHCII (clone 2G9) (BD Biosciences®). Samples were acquired on flow cytometer FACS CANTO II (BD Bioscience®). Data were analyzed using FlowJo software (TreeStar).

2.7. Annexin-V apoptosis assay

Apoptosis of BMDC was analyzed with BD Annexin V FITC apoptosis detection kit (BD Bioscience, San Jose, CA) following manufacturer's instruction. Cytometry was operated with a FACS Canto II (BD Bioscience).

2.8. Real-time PCR

Total RNA was extracted from RSV infected BMDC using Viral RNA/DNA Mini Kit (PureLink® - Invitrogen) following the manufacturer's instructions. cDNA was synthetized with random primers using Sensiscript® Reverse Transcription kit (QIAGEN®). The quality of the cDNA for each sample was tested by amplification of β-actin endogenous gene using specific primers and probes from TaqMan Assay (Applied Biosystems®). Samples that did not amplify β-actin gene were excluded. Real time PCR was performed to RSV protein F gene amplification using specific primers and probes (forward 5′-AACAGATGTAAGCAGCTCCGTTATC-3′, reverse 5′-CGATTTTTATTGGATGCTGTACATTT-3′ and probe 5′-FAM/TGCCATAGCATG ACACAATGGCTCCT-TAMRA/-3′). Data were analyzed by cycle threshold (CT). Delta CT value was calculated from the difference of the CT from the RSV protein F gene to the CT of β-actin gene. Fold-change in mRNA abundance was calculated using $2^{(-\Delta\Delta CT)}$ method (Schmittgen and Livak, 2008).

2.9. Statistical analysis

ANOVA test followed by a Bonferroni post-hoc test and *t*-test were applied to parametric data using Graph Pad Prism software (San Diego, CA, USA). Values demonstrated in graph are mean ± standard deviation and a level of significance of $p < 0.05$ was established for the analyses.

3. Results

3.1. Rapamycin treatment on BMDC decreases the frequency of $CD8^+$-$CD44^{high}$ T cells

In order to evaluate if mTOR inhibition on BMDC affects CD8 T cells differentiation during RSV infection, BMDC were treated with 20 ηg/mL of rapamycin before RSV infection and co-cultured with purified T cells. Our results demonstrated that rapamycin treatment on RSV-infected BMDC significantly decreased the frequency of $CD8^+CD44^{high}$ T cells (Fig. 1). Therefore, these data suggest that mTOR on DC is required to CD8 T cell differentiation during RSV infection, since its inhibition by rapamycin decreases the frequency of CD8 T cells with memory phenotype.

Rapamycin is known to diminish murine BMDC maturation, decreasing the expression of CD86, CD40 and MHC class II when these cells are treated during the differentiation process (Hackstein et al., 2003). We hypothesized that rapamycin treatment on RSV-infected BMDC diminishes $CD8^+CD44^{high}$ T cells frequency due to an impairment of BMDC activation. BMDC were differentiated during 6 days, treated with rapamycin, infected with RSV and after 96 h stained to evaluate the maturation markers by flow cytometry. We found that the percentage of $CD11c^+$ cells remains the same with the rapamycin treatment (Fig. 2A and B). In addition, the percentage of $CD11c^+$-$CD86^+MHCII^+$ cells is similar between the RSV-infected BMDC treated or not with rapamycin (Fig. 2C). These data suggest that rapamycin treatment on RSV-infected BMDC, in our conditions, does not affect the activation of BMDC since the expression of co-stimulatory molecule CD86 is preserved.

3.2. Rapamycin treatment on RSV-infected BMDC increases the RSV-RNA levels

We aimed to investigate whether rapamycin treatment affects RSV replication on BMDC and, consequently, the CD8 T cell differentiation. In order to evaluate that, RSV-RNA levels of infected rapamycin treated BMDC were analyzed by real time PCR after 96 h of infection. Rapamycin treated RSV-infected BMDC presented a significant 9-fold increase on RSV Protein F levels (Fig. 3).