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Original Articles

Immune activation, immune senescence and levels of Epstein Barr Virus in kidney transplant patients: Impact of mTOR inhibitors

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ABSTRACT

Post-transplant lymphoproliferative disorders (PTLD) represent a severe complication in transplanted patients and Epstein-Barr Virus (EBV) is the main driver. Besides immunodepression, immune activation/chronic inflammation play an important role in both virus reactivation and expansion of EBV-positive B cells. The aim of this study was to assess the impact of immunosuppressive strategies on factors involved in the PTLD's pathogenesis. 124 kidney transplanted patients were enrolled in this study: 71 were treated with mycophenolic acid (MPA) and 53 treated with mTOR inhibitor (mTORi), both in combination with different doses of calcineurin inhibitor. At the time of the transplant (T0), profile of inflammation/immune activation and immune senescence didn't differ between the two groups, but after one year of treatment (T1) markers were significantly higher in MPA-treated patients; their immunosenescence process was supported by the greater erosion of telomeres despite their younger age. Percentages of activated B cells and levels of EBV-DNA significantly increased in MPA-treated patients, and at T1 were significantly higher in MPA- than in mTORi-treated patients. Overall, these findings indicate that mTOR inhibitors constrain the inflammation/immune activation and senescence status, thus reducing the expansion of EBV-infected B cells and the risk of virus-associated PTLD in kidney transplant recipients.

1. Introduction

Post-transplant lymphoproliferative disorder (PTLD) is a potentially fatal complication of solid organ (SOT) and bone marrow transplantation. PTLD onset following SOT is estimated from 1% to 20% [1–3]. The highest rates of PTLD are observed in intestine, lung, and heart transplantation, while it is rare among kidney recipients (1%–5%); however, despite the relatively low incidence, kidney is the most frequently transplanted organ, making the overall number of PTLD in these patients high [4,5].

Epstein–Barr Virus (EBV) is the main driver of PTLD, particularly those occurring early after transplantation. Several factors concur to the PTLD development. The immunosuppression required to prevent graft rejection in transplant recipients causes a lack of effective control of viral replication and thus an increased risk of onset/progression of virally-induced cancers. In addition to protecting the host against oncogenic viral infections, an intact immune system prevents the establishment of a pro-tumorigenic inflammatory environment [6,7]. Pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) and 16S ribosomal (r)DNA, and damage-associated molecular patterns (DAMPs), such as mitochondrial DNA (mtDNA), can be released by early insults to the allograft that may accompany SOT [6,8,9]. Their binding to Toll-like receptors (TLRs) leads to the release of pro-inflammatory cytokines, such as interleukin (IL)-6, IL-10, and tumor necrosis factor (TNF)-α, causing a status of chronic inflammation that may activate EBV replication and/or expansion of EBV-infected B

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cells, thus increasing the risk of EBV-associated PTLD [6].

In renal transplant recipients, a regimen containing calcineurin inhibitors (CNI), such as tacrolimus, mycophenolic acid (MPA) and steroids is considered the standard of care. Long-term treatment with CNI is associated with adverse side effects such as nephrotoxicity, vascular disease, and increased risk of malignancy [10]. Mammalian target of rapamycin inhibitors (mTORi), such as everolimus, have been introduced in immunosuppressive regimens to prevent renal allograft rejection and are often used in the context of CNI-minimization protocols with the aim to reduce CNI-related renal and extra-renal toxicity and to avoid the hematological and gastrointestinal adverse events associated with MPA [11–13].

mTOR is a serine-threonine kinase that acts through mTOR complex 1 (mTORC1) and mTORC2; once activated, they regulate cell growth, proliferation, survival, autophagy, and metabolism. mTORi are inhibitors with several biological functions: there is emerging evidence that the anti-proliferative property of mTORi extends beyond lymphocytes and can inhibit the proliferation of cancer cells [12]. Controversial is their impact on PTLD incidence: some studies [14–16] found an association between the use of mTORi and a reduced risk of PTLD, while others found no association [3,17–19]. A recent study showed that mTORi rapamycin significantly suppressed growth of PTLD-derived tumor xenografts [20].

In this study we estimated the impact of two different immunosuppressive strategies (CNI and MPA *vs* CNI and mTORi) on pivotal factors, i.e. immune activation/inflammation, immune senescence, and expansion of cell-associated EBV load, driving the onset of PTLD.

2. Materials and methods

2.1. Patient characteristics

As part of thematic projects on virus-related and virus-unrelated cancer on kidney and liver post-transplanted patients, serial samples have been prospectively collected from patients who underwent kidney transplantation at the Kidney and Pancreas Transplantation Unit, Padova Hospital since January 1st, 2015. One-hundred twenty four kidney transplanted recipients who met the inclusion criteria were included in this study. Criteria of inclusion were: recipients of a first single/dual kidney transplantation from living or deceased donor. Criteria of exclusion were re-transplantation, combined kidney transplant with another solid organ, desensitization therapy pre-transplant due to AB0 or HLA-incompatibility, lymphocyte-depleting immunosuppressive therapies for acute rejection.

According to clinical practice, 71 received standard dose of CNI (tacrolimus 0.2 mg/kg/day with a trough level of 7–10 ng/mL for the first 3 months, then 5–8 ng/mL), MPA (Myfortic 720 mg bid), and steroids, and were defined as MPA-treated patients, and 53 received low dose of CNI (tacrolimus 0.1 mg/kg/day with a trough level of 3–5 ng/mL for the first year), mTORi (everolimus 1 mg bid, targeting a trough level of 3–5 ng/mL for all the duration of the study) and steroids, and were defined as mTORi-treated patients. As previously described [21], the use of mTORi in combination with low-dose CNI was introduced to offer an alternative to long-term full-dose CNI-based therapy to give a reduced renal toxicity, better cardiovascular profile and anti-proliferative effects, all of which would be of particular benefit in recipients of graft from extended criteria donors (ECD). Therefore, recipients of ECD graft were treated with a mTORi-based protocol, whereas recipients of standard criteria donors (SCD) received the MPA and full-dose CNI regimen. Based on an old-to-old allocation principle, recipients of kidneys from ECD are generally older than recipients of organs from SCD to avoid an unbalanced age mismatch [22]. All patients received immunosuppressive induction with Thymoglobulin (ATG) (started on day 0 with 2 mg/kg/day and continued until day 4 with the following dose regimen:1.5 mg/kg/day on day 1, 1.25 mg/kg/day on day 2, 1 mg/kg/day on day 3, 0.625 mg/kg/day on day 4) and the following steroid scheme (500 mg induction dose of methylprednisolone, tapered to reach 4 mg/day of prednisone at 2 months post-transplant). The study was approved by the Institutional Ethics Committee (CE 3262/AO/14) and conducted according to the Declaration of Helsinki and the guidelines of Good Clinical Practice. Written informed consent was obtained from all patients.

2.2. Sample collection

Blood samples from patients were taken in two EDTA-containing tubes at transplant (T0) and after one year of follow-up (T1). Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood by centrifugation on a Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient. PBMC were stained for flow cytometry analyses, and residual material was appropriately cryopreserved and stored in liquid nitrogen for PBMC and at - 20° or - 80 °C for plasma.

2.3. Flow cytometry analysis

Approximately 250,000 PBMC were stained for 15 min in the dark using the following labelled monoclonal antibodies (mAbs) (Becton-Dickinson, San Diego, CA, USA): anti-CD3 [fluorescein isothiocyanate (FITC)], anti-CD8 [peridinin chlorophyll protein (PerCP)], anti-CD38 [phycoerythrin (PE)], anti-HLA-DR [allophycocyanin (APC)], anti-CD279 (programed cell death 1, PD-1) [PE-Cy7], anti-CD28 [PE], anti-CD57 [allophycocyanin (APC)]; and anti-CD19 [V500], anti-CD10 [APC], anti-CD21 [FITC], anti-CD27 [PE-Cy7], anti-CD80 [APC-H7], anti-CD86 [APC], anti-CD274 (programed cell death ligand 1, PD-L1) [Brilliant Violet (BV) 421]. Appropriate isotype controls were used to evaluate non-specific staining. All the samples were analysed by LSR II cytofluorimeter (Becton-Dickinson). A total of 50,000 events was collected in the lymphocyte gate using morphological parameters (Forward- and Side-scatter). Data were processed with FACSDiva™ Software (Becton-Dickinson) and analysed using Kaluza® Analyzing Software v.1.2 (Beckman Coulter, Fullerton, CA, USA).

2.4. Quantification of microbial translocation and pro-inflammatory cytokines

DNA extraction from 200 µL of plasma was performed using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germania), and eluted in 50 µL of AE Buffer. A quantitative method based on real-time PCR assay was performed to quantify plasma levels of 16S rDNA with primer pair and probe as previously described [23]. Results were expressed as 16S rDNA copies/µL plasma. Plasma levels of lipopolysaccharide (LPS) were quantified with the Human Lipopolysaccharides ELISA Kit (Cusabio, Wuhan, China), and results were expressed as LPS pg/mL plasma. Plasma levels of mtDNA were quantified by real-time PCR assay with primer pair and probe as previously described [24]. Results were expressed as mtDNA copies/µL plasma. Levels of IL-10, IL-6, and TNF-α were quantified with Fluorokine MAP Human IL-6 kit, Fluorokine MAP Human IL-10 kit and Fluorokine MAP Human TNF-α/TNFSF2 kit (R&D Systems, Inc. Minneapolis, MN, USA) designed for using the Luminex 200TM, according to manufacturer's instructions.

2.5. Determination of telomere length

Relative telomere length (T/S) was determined by monochrome quantitative multiplex real-time PCR, as previously described [25].

2.6. EBV-DNA quantification

EBV type 1 and EBV type 2 in PBMC were quantified by a multiplex real-time PCR, as previously described [26]. The results were expressed as EBV-DNA copies/10^6 cells.

2.7. Statistical analyses

Continuous variables were summarized using median and interquartile range (IQR), categorical variables as frequencies and percentages. The association of patients' characteristics with the two different immunosuppressive strategies was assessed using the Mann-Whitney test and the $\chi 2$ or Fisher exact test as appropriate.

To evaluate the impact of CNI and MPA *vs* CNI and mTORi, the distributions of pivotal factors were compared between strategy groups at each time point by the Mann-Whitney test, adjusted for age and for age and CNI. Paired comparisons between time of transplant and one year of follow-up, within each group, were assessed by the Wilcoxon signed rank test.

To account for the multiple comparisons issue, p-values were adjusted applying the Benjamini-Hochberg correction. All statistical tests used a two-sided 5% significance level. Statistical analyses were performed using the RStudio (RStudio: Integrated Development for R. RStudio, Inc., Boston, MA), and the sanon package of R software (www.r-project.org/).

3. Results

3.1. Patients characteristics

One hundred twenty-four patients were enrolled in this study. The patients' characteristics at baseline are summarized in Table 1. Seventy-one (57%) were male. Median [interquartile range-IQR] age was 55 [46; 64] years; mTORi-treated patients were significantly older than MPA-treated patients (64 [57; 68] *vs* 50 [40;55] years, p < 0.0001).

According to clinical practice, the choice of CNI-minimization (mTORi-treated patients) was based on the characteristics of the donor, in order to reduce CNI exposure for those patients receiving a graft from an extended criteria donor [21]. ATG doses were similar between the two groups (4.9 [4.5; 5.4] *vs* 4.8 [4.0; 5.3] mg/kg, p = 0.0896).

Table 1
Baseline patients characteristics.

	all patients	CNI + MPA	CNI + mTORi	*p-value*
number	124	71	53	
Age at transplant				
Median [IQR]	55 [46–64]	50 [40–55]	64 [57–68]	< **0.0001**[a]
Gender				
male (%)	71 (57)	38 (54)	33 (62)	0.3303
female (%)	53 (43)	33 (46)	20 (38)	
Smoking				
neg (%)	92 (74)	53 (75)	39 (74)	0.8935
pos (%)	32 (26)	18 (25)	14 (26)	
Drinking				
neg (%)	122 (98)	69 (97)	53 (100)	0.5066
pos (%)	2 (2)	2 (3)	0	
Diabetes				
neg (%)	105 (85)	59 (83)	46 (87)	0.5721
pos (%)	19 (15)	12 (17)	7 (13)	
Dialysis				
neg (%)	13 (10)	8 (11)	5 (9)	1.0000
pos (%)	111 (90)	63 (89)	48 (91)	
Months in dialysis				
Median [IQR]	29 [13–59]	28 [12–65]	34 [16–55]	0.7979
IgG EBV anti-VCA				
neg (%)	2 (2)	2 (3)	0	0.5055
pos (%)	121 (98)	68 (97)	53 (100)	
IgG EBV anti-EBNA				
neg (%)	8 (7)	5 (7)	3 (6)	1.0000
pos (%)	115 (93)	65 (93)	50 (94)	
IgG CMV				
neg (%)	20 (16)	10 (14)	10 (19)	0.6224
pos (%)	104 (84)	61 (86)	43 (81)	

In bold p-value < 0.05.
[a] Manns-Whitney test.

Accordingly to the immunosuppressive scheme, mTORi-treated patients had lower trough levels of tacrolimus than MPA-treated patients (5.0 [3.7; 6.3] *vs* 9.9 [8.3; 12.2] ng/mL, p < 0.0001 at 7 day post-transplant; 4.2 [3.1; 5.3] *vs* 7.7 [6.8; 8.7] ng/mL, p < 0.0001 at T1). At 7 day post-transplant, s-creatinine levels were significantly lower in MPA-patients (127 [94; 248] *vs* 193 [135; 259] μmol/L, p = 0.0042); while at T1 they were similar between the two groups (116 [97; 135] *vs* 122 [101; 143] μmol/L, p = 0.3217) (Supplementary Table 1). All patients received standard antimicrobial prophylaxis (trimethoprim–sulfamethoxazole) for six months and ganciclovir during hospitalization, regardless of their cytomegalovirus antibody status. Acute rejections (in 4 MPA-treated and 2 mTORi-treated patients) were treated with pulse steroids. All patients were followed throughout the 12-months period.

3.2. Immunological profile: immune activation, senescence and checkpoints

At baseline (T0), the percentage of activated T lymphocytes were similar between MPA- and mTORi-treated patients (Fig. 1A and Supplementary Table 2). After one year of follow-up (T1), it significantly increased in MPA-treated patients (4.7 [3.3; 6.7] *vs* 9.5 [6.8; 15.3] %$CD3^+CD8^+CD38^+HLA\text{-}DR^+$, p < 0.0001), while decreased in mTORi-treated patients (6.8 [3.6; 12.5] *vs* 5.0 [3.1; 7.9] %$CD3^+CD8^+CD38^+HLA\text{-}DR^+$, p = 0.0027) (Fig. 1A). Moreover, at T1 the percentage of activated T cells was significantly higher in MPA-than mTORi-treated patients (Fig. 1A and Supplementary Table 2).

At T0, the percentage of immune senescent cells did not significantly differ between the two groups of patients (Fig. 2B and Supplementary Table 2). At T1, this percentage increased in both groups (4.2 [2.4; 7.9] *vs* 14.8 [11.4; 18.6] %$CD3^+CD8^+CD28^-CD57^+$, p < 0.0001 in MPA-treated patients, and 7.6 [5.4; 12.7] *vs* 13.1 [9.6; 18.2] %$CD3^+CD8^+CD28^-CD57^+$, p = 0.0519 in mTORi-patients) (Fig. 1B). However, the increase was more pronounced in the first group, so that at T1 the percentage of immune senescent cells was significantly higher in MPA- than in mTORi-treated patients (Fig. 1B and Supplementary Table 2).

At T0, PD-1 expression did not significantly differ between mTORi- and MPA-treated patients (Fig. 1C and Supplementary Table 2), and it did not significantly vary after one year follow-up in both MPA- (6.0 [3.9; 10.2] *vs* 7.4 [5.3; 9.9] %$CD3^+CD8^+PD\text{-}1^+$, p = 0.1677) and mTORi-treated patients (9.6 [6.9; 12.5] *vs* 7.5 [4.5; 11.4] %$CD3^+CD8^+PD\text{-}1^+$, p = 0.6781) (Fig. 1C). At T1, no difference has been found between the two groups (Fig. 1C and Supplementary Table 2).

At T0, activated B cells ($CD19^+CD80/86^+$) were similar between the two groups, while memory activated B cells ($CD19^+CD10^-CD21^-CD27^+$) were higher in mTORi- than MPA-treated patients (Fig. 1D and E and Supplementary Table 2). At T1, in MPA-treated patients both percentages of activated and memory activated B cells significantly increased (5.9 [3.9; 9.1] *vs* 11.5 [5.8; 15.4] %$CD19^+CD80/86^+$, p < 0.0001; 12.2 [7.6; 16.7] *vs* 16.2 [11.9; 25.9] %$CD19^+CD10^-CD21^-CD27^+$, p < 0.0001), while they decreased in mTORi-treated patients (8.5 [5.0; 12.0] *vs* 6.8 [5.0; 10.5] %$CD19^+CD80/86^+$, p = 0.1604; 13.9 [8.9; 22.3] *vs* 9.6 [5.9; 15.0] %$CD19^+CD10^-CD21^-CD27^+$, p = 0.0016) (Fig. 1D and E). At T1 the MPA-treated patients had higher levels of activated and memory activated B cells than those treated with mTORi (Fig. 1D and E and Supplementary Table 2).

At T0, the percentage of $PD\text{-}L1^+$ B cells did not significantly differ between the two groups (Fig. 1F and Supplementary Table 2); at T1, it significantly increased in MPA-treated patients (1.7 [0.8; 2.2] *vs* 5.5 [3.3; 7.5] %$CD19^+PD\text{-}L1^+$, p < 0.0001), while remained stable in mTORi-treated patients (1.4 [1.1; 2.2] *vs* 3.8 [1.5; 4.7] %$CD19^+PD\text{-}L1^+$, p = 0.2210) (Fig. 2F). At T1, percentages of $PD\text{-}L1^+$ B cells were significantly higher in MPA- than in mTORi-treated patients (Fig. 1F and Supplementary Table 2).

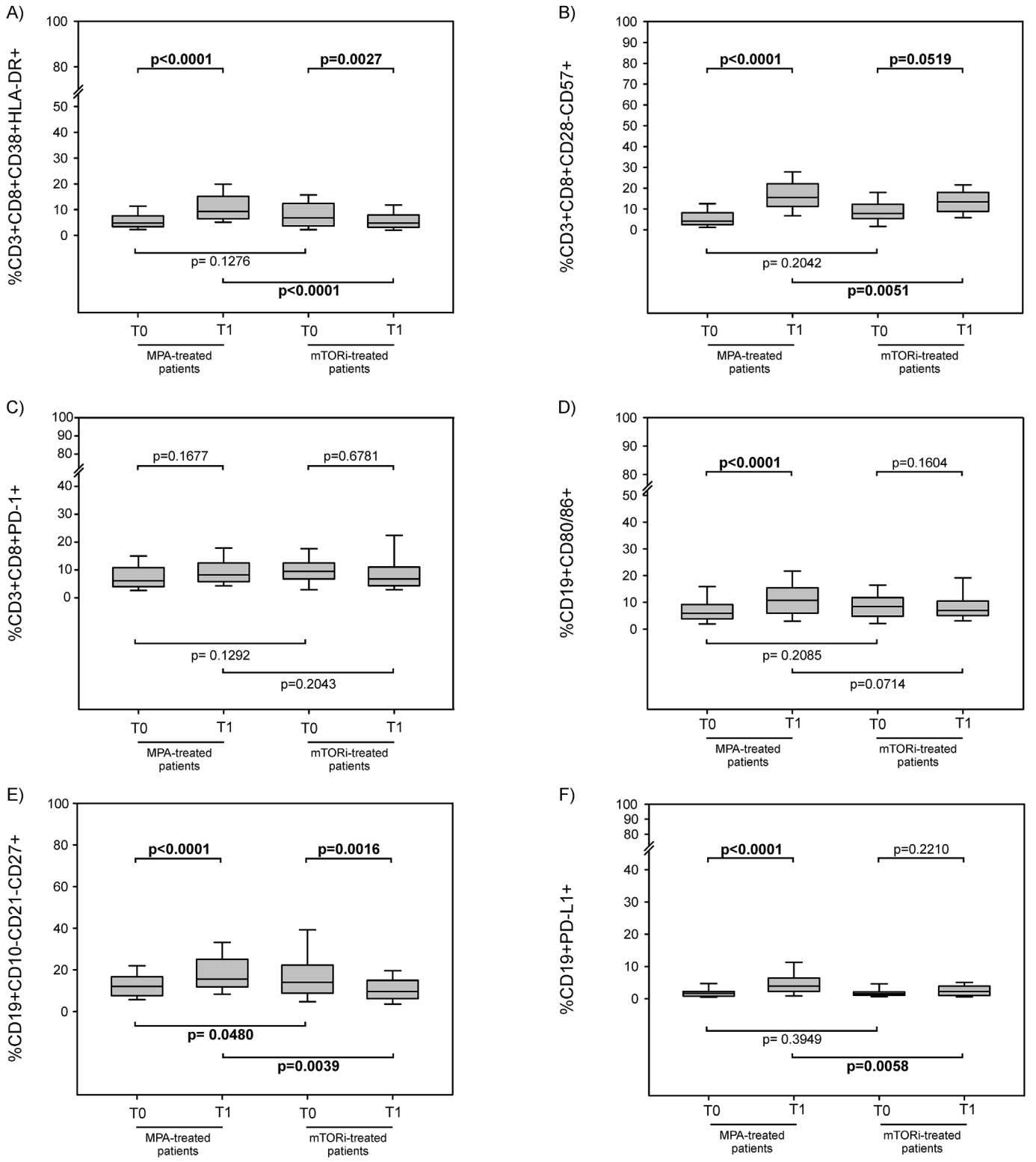

Fig. 1. Immune activation, senescence and checkpoints. Percentages of (A) activated ($CD3^+CD8^+CD38^+HLA\text{-}DR^+$), (B) senescent ($CD3^+CD8^+CD28^-CD57^+$), (C) programmed cell death (PD)-1 ($CD3^+CD8^+PD\text{-}1^+$) T cells, (D) activated ($CD19^+CD80/86^+$), (E) activated memory ($CD19^+CD10^-CD21^-CD27^+$), and (F) PD ligand 1 ($CD19^+PD\text{-}L1^+$) B cells in MPA-treated patients *vs* mTORi-treated patients at baseline (T0) and after one year of follow-up (T1).

3.3. Circulating markers of inflammation: PAMPs, DAMPs and cytokines

At T0, the circulating levels of PAMPs did not significantly differ between the two groups of patients (Fig. 2A and B and Supplementary Table 2). At T1, levels of 16S rDNA and LPS significantly increased in the MPA-treated patients (40 [10; 71] *vs* 62 [31; 103] 16S rDNA copies/μL, $p < 0.0001$; 105 [64; 144] *vs* 140 [102; 179] LPS pg/mL, $p = 0.0118$), while remained stable in mTORi-treated patients (34 [10; 59] *vs* 26 [10; 76] 16S rDNA copies/μL, $p = 0.8560$; 98 [65; 137] *vs* 131 [52; 144] LPS pg/mL, $p = 0.2751$) (Fig. 2A and B). At T1, levels of PAMPs were higher in MPA-than in mTORi-treated patients (Fig. 2A and B and Supplementary Table 2).

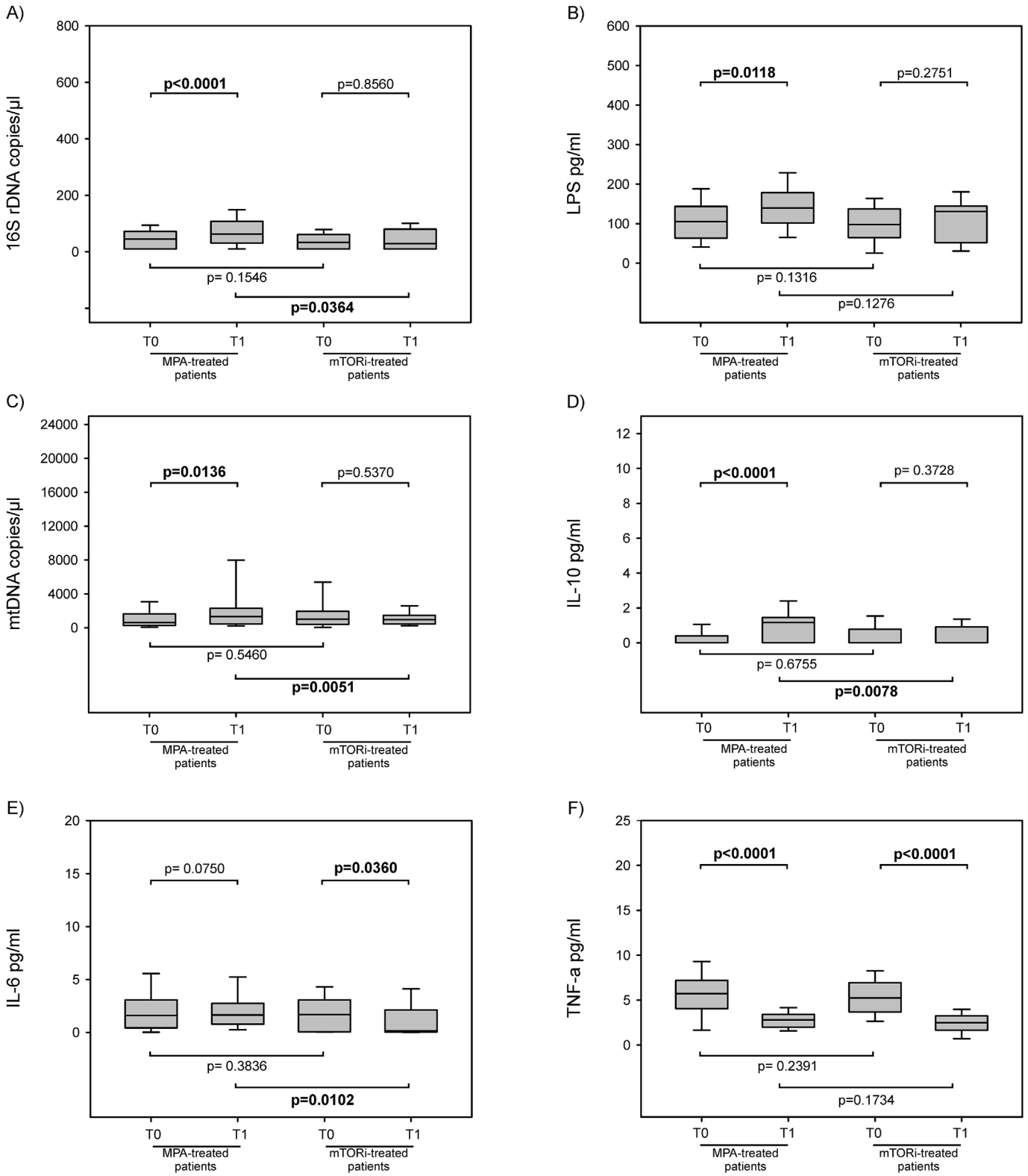

Fig. 2. Circulating markers of inflammation: PAMPs, DAMPs and pro-inflammatory cytokines. Circulating levels of PAMPs [(A) 16S rDNA and (B) LPS], DAMPs, [(C) mtDNA] and pro-inflammatory cytokines [(D) IL-10, (E) IL-6, and (F) TNF-α] in MPA-treated patients *vs* mTORi-treated patients at baseline (T0) and after one year of follow-up (T1).

A similar trend was observed for mtDNA, a marker of DAMPs. Its circulating levels did not differ between the two groups at T0 (Fig. 2C and Supplementary Table 2). At T1, mtDNA was significantly increased in MPA-treated patients (640 [282; 1670] *vs* 1228 [372; 2235] mtDNA copies/µL, p = 0.0136), and was significantly higher than in mTORi-treated patients (1331 [484; 2235] *vs* 976 [471; 1481] copies/µL, p = 0.0051), where remained stable (Fig. 2C and Supplementary Table 2).

At T0, circulating levels of all evaluated pro-inflammatory cytokines were similar between the two groups (Fig. 2D, E, F and Supplementary

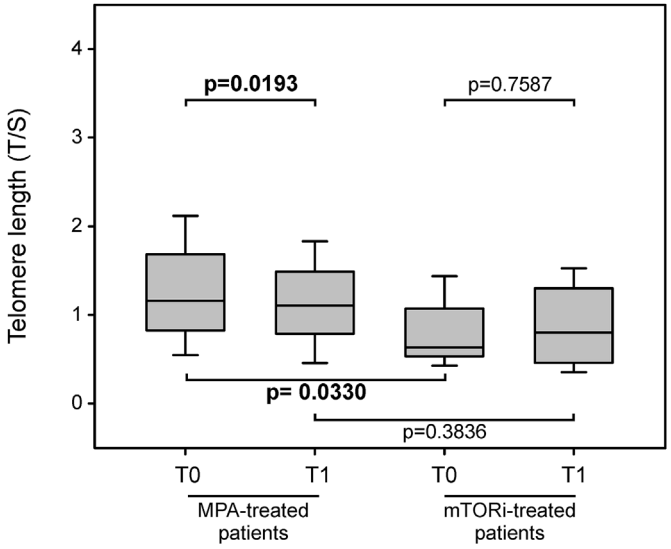

Fig. 3. Telomere length. Telomere length in MPA-treated patients *vs* mTORi-treated patients at baseline (T0) and after one year of follow-up (T1).

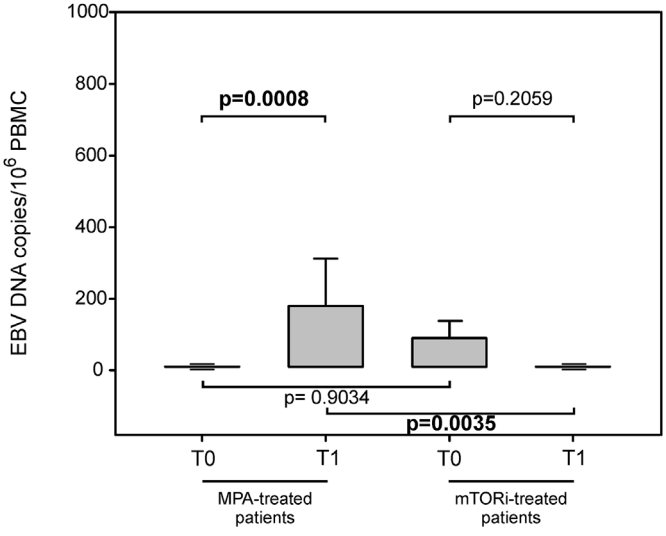

Fig. 4. Levels of EBV-DNA. EBV-DNA levels in MPA-treated patients *vs* mTORi-treated patients at baseline (T0) and after one year of follow-up (T1).

Table 2). At T1, IL-10 levels were significantly increased in the MPA-treated patients (0.0 [0.0; 0.5] *vs* 1.2 [0.0; 1.5] IL-10 pg/mL, $p < 0.0001$), while they did not significantly vary in mTORi-treated patients (0.0 [0.0; 0.8] *vs* 0.0 [0.0; 0.9] pg/mL, $p = 0.3728$) (Fig. 2D). IL-6 levels did not significantly differ in MPA- (1.6 [0.4; 3.1] *vs* 1.7 [0.8; 2.8] pg/mL, $p = 0.0750$), while they significantly decreased in mTORi-treated patients (1.7 [0.0–3.2] *vs* 0.2 [0.0; 2.2] pg/mL, $p = 0.0360$) (Fig. 2E). About TNF-α, its levels significantly decreased in both group (6.1 [4.7; 8.2] *vs* 2.8 [1.9; 3.5] pg/mL, $p < 0.0001$ in MPA-treated patients; 5.5 [4.3; 6.9] *vs* 2.4 [1.7; 3.1] pg/mL, $p < 0.0001$ in mTORi-treated patients) (Fig. 2F). At T1, IL-10, IL-6 and TNF-α were higher in MPA- than in mTORi-treated patients (Fig. 2D, E, F and Supplementary Table 2).

3.4. Telomere length

The telomere length (T/S), a marker of biological senescence, was measured in the peripheral blood cells of patients. At T0 the median T/S value was significantly higher in MPA- than in mTORi-treated patients (1.2 [0.8; 1.7] *vs* 0.6 [0.5; 1.1] T/S, $p = 0.0330$) (Fig. 3 and Supplementary Table 2). Clinical characteristics that may contribute to telomere attrition, as smoking [27], diabetes [28] and dialysis [29], did not differ between the two groups, thus lower telomere length of mTORi-treated patients seems to be mainly due to their older age. Notably, after one year follow-up, telomere significantly shortened in MPA-treated patients (1.3 [0.7; 1.8] *vs* 1.1 [0.8; 1.5] T/S, $p = 0.0193$), while no significant variation did occur in telomere length of mTORi-treated patients, and at T1 no difference was found between the two groups (Fig. 3 and Supplementary Table 2). Thus, the erosion of telomere in MPA-treated patients was significantly higher than that observed in mTORi-treated patients (-0.1 [-0.4; 0.2] *vs* 0.0 [-0.1; 0.2] ΔT/S, $p = 0.0392$).

3.5. EBV-DNA levels

At T0, levels of EBV-DNA were similar in the two groups of patients (Fig. 4 and Supplementary Table 2). Levels of EBV-DNA significantly increased in MPA-treated patients (10 [10;10] *vs* 10 [10; 210] copies/10^6 PBMC, $p = 0.0008$), while remained stable in mTORi-treated patients (10 [10; 90] *vs* 10 [10;10] copies/10^6 PBMC, $p = 0.2059$) (Fig. 4). Notably, at T1 MPA- had higher levels of EBV than mTORi-treated patients (Fig. 4 and Supplementary Table 2).

4. Discussion

The combination use of mTORi with low doses of CNI was investigated and its efficacy on renal function was already demonstrated in kidney transplant patients, compared to the standard care (CNI and MPA) [13,30–33]. Little was reported on the impact of this two different treatment (MPA/CNI *vs* mTORi/CNI) on viral and host factors involved in the mechanisms of PTLD pathogenesis, that represent a severe complication in kidney transplanted patients.

Events associated with clinical transplantation, such as the ischaemia-reperfusion injury (IRI) and/or the immunosuppressive treatment itself may induce a damage of epithelial barrier, allowing the release of DAMPs and PAMPs into circulation [6,8,9,34]. Both DAMPs and PAMPs initiate a cascade of signaling that induce the production of pro-inflammatory cytokines, establishing a state of chronic inflammation. It is known that mTORi have an anti-inflammatory effect [35]: *in vitro* studies demonstrated that the inhibition of mTORC1 impairs the production of several pro-inflammatory cytokines [36,37]. Moreover, it has been recently found that everolimus has an inhibitory effect on IL-2, IL-10, IL-21 and IFN, reducing their mRNA expression, also in combination with low levels of tacrolimus, while MPA has no inhibitory effect on any of these cytokines [38]. Our results confirm the anti-inflammatory effect of mTORi treatment. Conversely to patients under MPA-based therapy, mTORi-treated patients did not show an increase of DAMPs and PAMPs, and this may explain also their lower levels of pro-inflammatory cytokines. The impact of old age on augment of pro-inflammatory transcripts, including cytokines [39], and of different levels of CNI were excluded by adjustment for age and CNI levels (Supplementary Table 2).

The mTOR pathway is a critical regulator of immune function in promoting differentiation, activation, and function of T and B cells [35,40,41], and everolimus has been described as a potent inhibitor of T- and B-cell activation [12,41,42]. In our study we found that, conversely to MPA-treatment, where immune activation was significantly increased after one year of follow-up, in mTORi-treated patients the percentages of activated T and B cells decreased, very likely through the lower release of pro-inflammatory cytokines.

Aging is a physiologic/pathologic process characterized by a progressive impairment of cellular functions, supported by the alteration of several molecular pathways, leading to an increased cell susceptibility to injury [43]. mTOR is considered a crucial modulator of aging [43], and it has been demonstrated that inhibition by rapamycin might prevent the induction of the senescence associated secretory phenotype

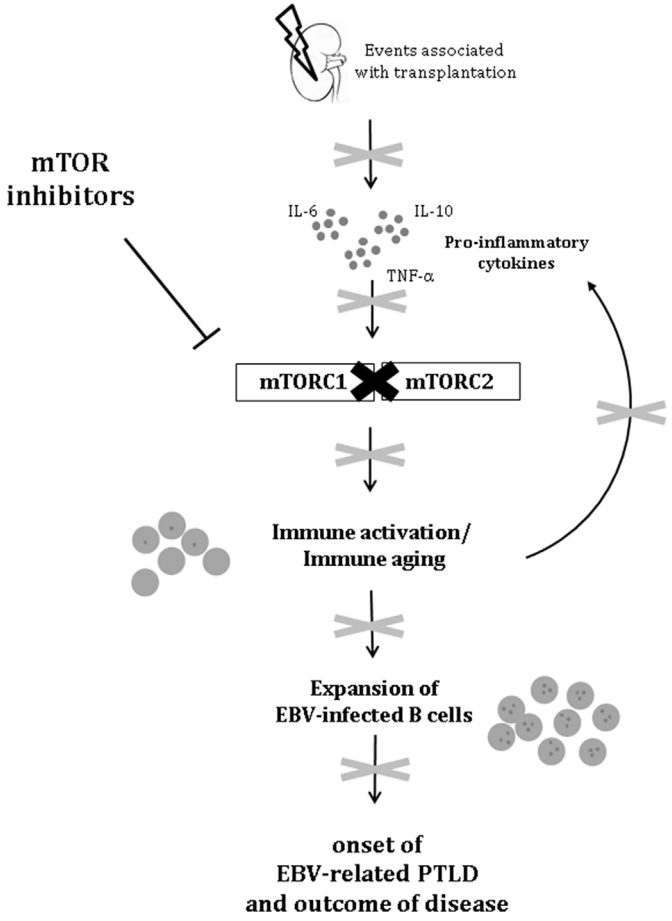

Fig. 5. Potential impact of mTOR inhibitors on the factors involved in the pathogenesis of EBV-related PTLD. Events associated with transplantation, such as the ischaemia-reperfusion injury and/or the immunosuppressive treatment, induce an inflammatory status. The production of pro-inflammatory cytokines leads to the activation of mTOR pathway (mTORC1 and mTORC2), that is crucial to promote the immune activation and immune aging, which in turn contribute to increase the pro-inflammatory cytokines levels. The persistent inflammation/immune activation may also favor the expansion of EBV-infected B cells, a pivotal step for the EBV-related PTLD development. Inhibition of mTORC using mTORi (black cross) restricts (grey cross) immune activation, that in turn down-regulates the levels of inflammatory cytokines, and the EBV-positive activated B cells, thus reducing the risk of EBV-related PTLD.

(SASP) [44–46]. In this study, we found that MPA-treated patients had higher level of immune senescent cells than mTORi-treated patients. In agreement with this observation, MPA-treated patients showed, during one year follow-up, a greater erosion of telomere length compared to mTORi-treated patients, despite the younger age of the former. Within this frame, literature reported a greater telomere attrition in renal transplanted patients treated with mycophenolate mofetil (an analogous of MPA) [47], while it was observed that telomere shortening was less severe under treatment with rapamycin [48,49]. Overall, our findings suggest that the use of mTORi may inhibit the immunosenescent process, reducing the SASP and the consequent release of pro-inflammatory cytokines, thus inhibiting the inflammaging phenomenon.

Literature extensively reported that increased EBV-DNA load is a strong predictive risk factor for the onset of PTLD and assessment of EBV viremia is of clinical importance in transplant recipients, representing a useful tool for early diagnosis and monitoring of patients at high risk for PTLD [6,50]. Little was reported about the impact of mTORi on EBV [51]. A retrospective study conducted in 19 heart transplant patients demonstrated that the switch from CNI to mTORi was followed by a decrease in EBV load, and a lower risk of PTLD recurrence [52]. In a case report of an elderly kidney transplant recipient who developed EBV-positive monomorphic T-cell PTLD, the conversion from conventional immunosuppressive strategy to everolimus induced complete remission of PTLD accompanied by a decrease in blood EBV-DNA level [53]. Here, we found that, while EBV levels increased in the MPA-treated group, EBV levels did not increase in mTORi-treated patients. Contextualizing these data in the pathogenic mechanism of PTLD strengthened the finding that the use of immunosuppressive therapy based on mTOR inhibitors reduces the stimulation of B lymphocytes and the expansion of B cells infected with EBV, with consequent persistence of low viral levels and low risk of the PTLD onset. Notably, in this study patients were followed for twelve months and no case of PTLD was observed during this short period. However, a previous epidemiological study conducted in our and other Italian kidney transplant centers suggests a protective effect of mTORi in reducing the risk of post-transplant cancers, including PTLD [16].

The PD-L1, together with PD-1, is recognized to be an important central checkpoint in the immune invasion for tumor progression [54], and PD-L1 positive cells were found in EBV-positive PTLD specimens [55]. It has been described that PD-L1 expression, activated by EBV-encoded protein LMP-1 through JNK cascade, contributes to lymphomagenesis in EBV-driven tumors [55,56]. Furthermore, increased levels of cytokines (IFN-γ and IL-10) promoted PD-L1 expression [54]. In this study, we found that B cells expressing PD-L1 were significantly increased in MPA-treated patients, who showed also an increased level of IL-10. Finding that the percentage of PD-L1-positive B cells did not increase in mTORi patients may suggest that inhibition of mTOR has the potential to control PD-L1 signaling on lymphomagenesis.

In conclusion, our findings suggest that mTOR inhibitors may better control the condition of chronic immune activation/inflammation, reducing the production of pro-inflammatory cytokines, and immune senescence in kidney transplant recipients, with a consequent better control on the expansion of EBV-positive B cells, which limits the increase of EBV load (Fig. 5). Overall, these findings suggest that mTORi may play an important role in reducing the risk of PTLD associated with EBV.

Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

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