Perforin: An intriguing protein in allograft rejection immunology (Review)

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Received September 3, 2021; Accepted October 5, 2021

DOI: 10.3892/etm.2022.11446

Abstract. Chronic kidney disease (CKD) is a worldwide public health problem. The constantly increasing prevalence of CKD requires further research into new additional strategies in its management. The preferred treatment of end-stage renal disease (ESRD) is renal transplantation. Kidney transplant patients benefit from substantial improvement in their quality and duration of life. For these to be feasible, the long-term graft and host survival optimization of the renal transplant recipient must be ensured and chronic allograft dysfunction (CAN) must be prevented. Once an equilibrium in the allograft tolerance is established, renal transplanted patients would benefit from the withdrawal or the reduction of immunosuppression therapy. Identification of early predictive biomarkers of CAN is essential. Recent publications have revealed that in long-term immune tolerance and graft survival several populations of immune cells are involved. Starting from the identification of perforin (PRF) in pathological renal glomeruli and following with the analysis of the molecular expression of PRF in renal biopsy samples, it appears that serum PRF is one of the potential biomarkers of graft dysfunction. Over the years, this protein has captured the attention of the medical world, conducting research that could potentially lead to the discovery of an innovative biomarker. Discovering and understanding the involvement of PRF in developing CAN may open up new therapeutic pathways that would ensure the survival of the kidney transplant. In this review the authors examined the structure, the role and the present understanding of the mechanisms by which serum PRF may be involved in chronic graft dysfunction as well as its role as an immune tolerance biomarker for chronic dysfunction of the renal graft.

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Key words: transplantation, perforin, granzyme, graft dysfunction, allograft rejection

Contents

1. Kidney transplantation in chronic kidney disease

Chronic kidney disease (CKD) is a worldwide public health problem. The constantly increasing prevalence of CKD requires more research into new additional strategies in its management. Impaired kidney function is accompanied by numerous complications related to water and electrolyte balance disorders and accumulation of uremic toxins which are physiologically excreted in the urine, as well as increased risk of cardiovascular events, thus affecting mortality, morbidity and the quality of life of patients with CKD (1).

The prognosis of CKD patients is dependent on the progression of renal dysfunction to its total function loss and also, on the specific complications of their chronic disease (2-4).

The optimal treatment for renal function impairment is kidney transplantation, which ensures a higher quality of life and longer survival than maintenance with dialysis, in patients with end-stage renal disease (ESRD). Over the last few years the prognosis of kidney transplanted patients has significantly improved reaching a graft survival rate of over 92% per year (5-7). Currently, the attention is directed on prolonging long-term graft survival as much as possible.

A narrative review was performed on articles published between 1980 and 2020, which were identified on PubMed, via specific mesh terms: 'Perforin', 'kidney', 'granzyme', 'transplantation', 'graft dysfunction'. Furthermore, citation tracking of the studies retrieved was used to identify additional relevant articles. Only the articles written in English were evaluated. A total of 93 references were introduced.

2. Brief immunology of renal transplantation

Transplant rejection represents the rejection of a transplant allograft or transplanted organ. This occurs because the graft is accompanied by a series of antigens that the immune system of the recipient perceives as non-self, and consequently an immune response (host vs. graft reaction) is produced (8).

The mammalian immune system is an extremely complex system developed over millions of years as evolved immune response of vertebrates against microbial invasion and ensures the species continuity. The system could be divided into adaptive and innate immunity. Innate immunity represents a non-specific immune system, and the first line of defense that involves recruitment and participation of macrophages, neutrophils, natural killer (NK) cells, cytokines, certain cell receptors and complement components and precedes adaptive immunity functioning as secondary signals for lymphocyte activation (8).

While the inherited immunity does not involve the recognition of specific antigens, adaptive immunity involves the recognition of a wide range of molecules, the identification of different similar structures (high specificity to the pathogen agent) and the immune memory (recognition of the aggressor at the first contact and specific reaction by an accelerated and protective response) (9). The adaptive immune response is considered the most important hurdle in organ transplantation.

The main target of the immune response to the graft in organ transplantation are the major histocompatibility complex molecules (MHC) expressed on the surface of the donor cells; this feature is a form of adaptive immunity (9). The MHC is a complex of polymorphic genes encoded in a locus situated on the short arm of human chromosome 6. MHC protein products are expressed on the surfaces of various cells. In humans these are called human leukocyte antigens (HLA) and are analogous to the H-2 (in mice) and RT1 (in rats) systems (9). Graft antigens that serve as the main target of rejection are proteins encoded by the MHC genes.

Graft rejection is the result of immune mechanism activation due to antigenic differences between MHC I and II molecules of the recipient and donor, the latter acting as major antigens in the body of the recipient. These molecules present a high polymorphism, in particular those of HLA class I A and B with at least 200 and 250 alleles, respectively, which have been described in the human population while HLA-C and HLA-DP have a limited polymorphism and thus low significance (9,10).

In order to have moderate affinity for their own MHC molecules, specifically selected T cells during thymus development recognize portions of protein antigens that have been fragmented into peptides bound to MHC class I and II molecules. T-cell recognition of the antigen is the main event that initiates the effect of immune response mechanisms followed by two discrete signals. The first phase (signal 1) is the recognition of the complex formed by the MHC class II molecule and the antigenic peptide by the surface lymphocyte receptor (TCR-CD3) and Th (T helper) fixing to the antigen-presenting cell (APC). The T-cell receptor is a heterodimer consisting of an α polypeptide chain and a β polypeptide chain, which associates on the surface of the T cell, with the CD3 polypeptide complex. Signal 2 is received by the CD28 accessory molecules that bind to the B7-1 (CD80) or B7-2 (CD86) molecule on the APC surface (11,12).

Once activated T cells undergo proliferation under the influence of mitogenic growth and differentiation factors such as interleukin (IL)-2 and IL-5, which activate the target of rapamycin (TOR) paths; this process requires nucleotide synthesis. Cell proliferation and differentiation induce cytotoxicity mediated by the lymphocytes $CD8^+$ T, activating B lymphocytes (either directly or depending on Th lymphocytes) to produce antibodies and determine macrophages to induce delayed hypersensitivity responses (11,13) (Fig. 1).

Detailed studies of these steps have led to the development of targeted immunosuppressive therapies such as IL-2 receptor blockers (basiliximab), mTOR inhibitors (sirolimus and everolimus), nucleotide synthesis inhibitors (mycophenolate) or antimetabolites (azathioprine) (14).

In 2014, a new series of small molecules with inhibiting PRF function was tested on male CD1 mice after intravenous administration; the compounds exhibited microsomal stability, which may lead to the development of a new immunosuppressive therapy (15). In contrast, the induction of PRF mRNA was partially blocked by the immunosuppressive drug cyclosporine A, and therefore this therapy has been recently avoided due to toxicity in favor of tacrolimus administration (16).

3. PRF-granzyme pathway

The main mechanism used by lymphocytes for eliminating infected or malignant cells in the host involves granule exocytosis which contains PRF and a family of serine esterases known under the name of granzymes. The fundamental basis of the apoptotic pathway PRF-granzyme, is the synergy between its components PRF and granzymes. These molecules have distinct roles. PRF pores serve as entrance gates for the protease in the targeted cell cytosol allowing granzymes to initiate various apoptotic pathways. Although granzymes can internalize independent of PRF, when seized into the endocytic vesicle lumen, they do not have access to cytosolic substrates and remain harmless. Thus, the expression of PRF is required for granule-mediated cytotoxicity, ensuring the entry of granzymes into the targeted cells, the latter to induce apoptosis (17-20) [Fig. 2, PRF-granzyme B pathway adapted from (12); T-cell immune response through the release of PRF and granzyme B, which attack target cells, inducing apoptosis.].

PRF mRNA was identified in $CD8^+$ cells infiltrating the glomerulus of crescentic glomerulonephritis rats. In human crescentic glomerulonephritis, both CD4- and CD8-positive T lymphocytes are observed in glomeruli (21).

4. Chronic allograft dysfunction

Despite major advances in immunosuppression and transplant management, acute and chronic rejection are the main causes of kidney graft loss. It was revealed that acute rejection is the strongest predictor of subsequent chronic rejection (22).

CAN occurs due to repeated episodes of acute rejection, HLA system lack of compatibility, improper

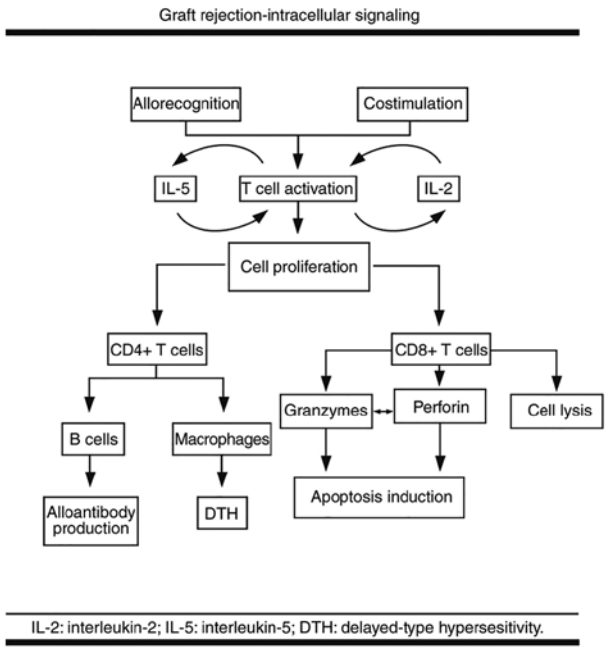

Figure 1. Graft rejection-intracellular signaling adapted by Ref (13). Cell proliferation under mitogenic growth and differentiation factors of which the best characterized are IL-2 and IL-5; this leads to cytotoxicity mediated by $CD4^+$ and $CD8^+$ T cells which activates B lymphocytes to produce antibodies and determine macrophages to induce delayed hypersensitivity responses.

immunosuppression, ischemic injury (vascular occlusion caused by arterial immune-mediated thickening and dysfunction) with secondary fibrosis and late recovery of renal function (23).

Early allograft lesions by the PRF-granzyme pathway could initiate the development of CAN in renal allografts (24). $CD4^+$ Th lymphocytes reactive to graft alloantigens produce cytokines which induce endothelial and smooth muscle cell proliferation and cause occlusion of the vascular lumen. It appears that cytotoxic T lymphocytes infiltrated in the renal graft induce allogeneic tubular epithelial cell death, via the native PRF pathway (25,26).

The diagnosis of chronic rejection is not always made in a timely manner, required for the complete recuperation of renal function. This problem occurs since kidney damage is detected mainly as an increase in serum creatinine and the appearance of proteinuria. However, an increase in serum creatinine is a late sign of kidney damage, as the compensatory mechanisms in the kidneys can maintain the glomerular filtration rate (GFR) despite progressive structural damage. Although the long-term survival of allografts is improving, late graft loss from CAN remains a clinically significant problem and is the second most common cause of late renal allograft loss, after death (27). Thus, the identification of early markers associated with, or predicting CAN would be clinically useful.

Recurrent or chronic inflammatory processes are common in people with CKD and particularly in those with ESRD. This is due to numerous underlying factors including the uremic environment, high levels of circulating proinflammatory cytokines, oxidative stress, carbonyl stress, waste of protein energy (PEW), and increased incidence of infections (especially dialysis access) to mention a few (28).

The acute-phase response is a major pathophysiological phenomenon that accompanies inflammation. With this reaction, normal homeostatic mechanisms are replaced by new established factors that are likely to contribute to defensive or adaptive capabilities (29,30).

In patients returning to a dialysis program after acute or chronic rejection of the renal transplant but without kidney transplant graftectomy performed, a chronic inflammatory state was observed, that was reduced by the removal of the non-functional graft (explant) (31).

5. Predictive markers of chronic allograft dysfunction

To date, few studies have been performed on PRF in the renal area. In an experimental study, using Wistar-Kyoto (WKY) rats with antiglomerular basement membrane (GBM) crescentic glomerulonephritis (GN), PRF protein and mRNA expression of PRF were demonstrated in glomeruli by immunohistochemistry and *in situ* hybridization. WKY rats treated with anti-PRF antibodies revealed significantly reduced amounts of proteinuria and frequency of crescentic glomeruli (21).

There is strong scientific evidence that immunological non-invasive monitoring could be useful in the first 6 months after kidney transplantation in particular regarding prediction of acute rejection episodes (32). It is less clear whether CAN is also associated with consistent changes of peripheral blood or the urinary cells. Several histological studies have demonstrated enhanced expression of granzymes and PRF in numerous types of transplanted grafts and their correlation with acute rejection episodes (33-40). It appears that the urinary mRNA levels of three markers including PRF, granzyme B and FAS ligand appear to be correlated with acute rejection and the increase of serum creatinine (41,42). The molecular analysis of the expression of these three molecules in renal biopsy samples revealed that only the expression of PRF and FAS ligand were correlated with the acute rejection while the expression of PRF and granzyme B could intensify at a longer time after transplantation, possibly associated with chronic dysfunction (43). In 1997, a concurrent RT-qPCR assessment of PRF, granzyme B and Fas ligand revealed a correlation with acute rejection even in cases of mild infiltration, with 100% sensitivity and specificity. The combined analysis of the expression of Fas ligand, PRF and granzyme B genes by quantitative RT-PCR provided a reliable tool for the diagnosis and management of acute renal rejection and antirejection therapy leading to a rapid decrease in the expression of these genes (44). Li *et al* confirmed that the mRNA levels of PRF and granzyme B were increased in urine samples from patients with acute rejection (42). To date, it has been demonstrated that urinary mRNA levels of AGT, EGFR and TGF-β1 may be reliable prediction markers of CAN (32,45-47).

The genetic expression of serum PRF has been revealed to be more correlated with acute rejection in renal transplanted patients in comparison with granzyme B and Fas ligand thus supporting its use as a marker of acute rejection (48). The PRF-granzyme and the Fas ligand are two major pathways by which cytotoxic T lymphocytes induce apoptosis in target cells (49). The expression of the message into the graft for these

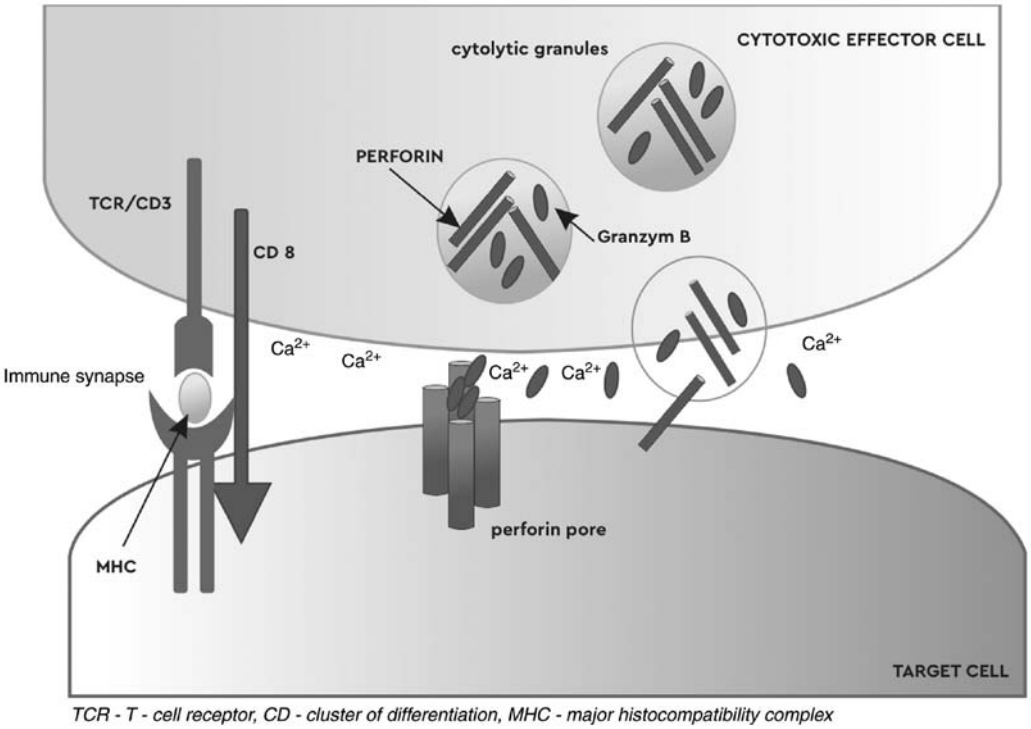

Figure 2. PRF-granzyme B pathway adapted from Ref (12). T-cell immune response through the release of PRF and granzyme B, which attack target cells, inducing apoptosis. PRF, perforin.

immune-activating genes has been revealed to be markedly correlated with graft rejection. In a retrospective pilot study, 140 fine-needle aspiration biopsy samples from 50 human renal allografts were labeled using alkaline phosphatase/alkaline anti-phosphatase immunocytochemistry incorporating monoclonal antibodies to PRF, granzyme B, and Fas ligand. Positive labeling levels for these markers were compared with the initial clinical diagnosis of rejection. Only when all three antibodies yielded positive labeling, was the association with the clinical rejection status superior to conventional morphological cytology (50).

A recent study on humanized mice, revealed that granzyme B expression was significantly increased in $CD8^+$ T cells in patients with graft rejection, while surviving graft patients expressed less granzyme B, as they had an increased level of HLA-G dimer which inhibited cytotoxicity of $CD8^+$ T cells (51). The synergy between PRF and granzymes is already established. Therefore, a high level of granzymes implies a high level of PRF.

Recently, new categories of drugs, such as inhibitors of PRF, have aroused the interest of researchers. Tampio *et al* demonstrated that using L-type amino acid transporter 1 (LAT1)-utilizing prodrugs of PRF inhibitors for improved administration of brain drug delivery, led to improved pharmacological effects, decreased production of cellular apoptosis mediators, decreased overall oxidative stress and inflammation in the brain, and from the periphery, increasing cell survival (52).

6. PRF 'roots'

In the evolution of PRF there were complex models of events of birth and death including duplication/pseudogenization to mammals, multiple amplifications and losses in reptiles and fish as well and a case of partial duplication with a new beginning codon to fish. Approximately 500 million years ago, the primordial PRF gene evolved, around the same time as T-cell receptor antigen recognition based on the major histocompatibility complex. As it is absent from primitive chordates and invertebrates, cytotoxic cells from these lineages must have a different cytotoxic effector molecule or mechanism. Orthologs and homologues of human PRF have been identified in almost all species. Research has shown that in species prior to Gnathostomata (Euteleostomi) the PRF gene did not exist which suggests that cytotoxic cells of prior species have another mechanism or different means for killing targeted cells. In addition, there is evidence that PRF originated from the duplication of the ancient gene MPEG1 and shares a common ancestor with functionally related complement proteins (53,54).

7. PRF: Structure and genetics

PRF is a 67-kDa pore-forming protein, stored and released from the secretory granules (SG) of the cytotoxic lymphocytes which leads to osmotic lysis of the membrane of target cells and subsequently allows proapoptotic granzymes (serine proteases which split the peptide connections of proteins) with broad specificity to enter the targeted cells and activate the cell death program. PRF expression is increased in the activated $CD8^+$ cells, $\alpha\gamma$ T cells, in subpopulations of activated $CD4^+$ T cells, and NK cells (but with a high and stable incorporation in NK cells) (17,19,55-58). In addition, PRF expression may be stimulated in some activated $CD4^+$ cells (59,60).

In mammals, PRF is encoded by the PRF1 gene expressed in cytotoxic lymphocytes and regulatory T cells. PRF1 transcription is the main mechanism that determines PRF expression in cytotoxic T lymphocytes and NK cells. While PRF is uniformly expressed by mature NK cells as a result of spontaneous stimulation of constitutive gene transcription, its expression in peripheral T cells requires gene activation (17,58,61-64).

Locus control position is essential for PRF1-specific activity (NK and cytotoxic cell activation). A heterochromatin-dependent regulation could allow certain exogenous stimuli and certain endogenous controlling of the transcription factors to induce PRF1 transcription in other types of cells (59). In 2006, a study conducted by Pipkin and Lichtenheld identified the locus control region for perforin of 150 Kb of *cis* action sequences which leads to the physiological PRF1 transcription, comprising 16 hypersensitive DNase I (DHS) sites, four of them necessary for PRF expression (17,65). PRF was identified for the first time in 1983 and it was cloned from an expression library by a cross reaction of the C9 antibody (59,66-71). Fine-resolution comparisons by direct sequence comparisons have revealed a similarity between the two proteins (C9 and PRF), that contain in the middle part of their sequences a short region called membrane-attack complex/PRF (MACPF) (59,72,73). Both proteins polymerize in tubular complexes able to determine lysis of the membrane insertion acting as large and voltage-independent transmembrane channels. Initial studies have revealed that while C9 polymerizes in physiological conditions requiring the assembly of complex C5b-8 into the receptor, the functional activity of PRF in the phospholipid membrane is calcium-dependent (67,74,75). After exocytosis, granules from the killer cells releasing PRF and granzymes are exposed to immune synapses rich in calcium and neutral pH (59,64). The PRF monomers bind to calcium by its C2 domain acquiring the capacity of bounding lipids to the targeted cell membrane and then to merge in transmembrane pores of up to 100 Å, which allows granzymes access to the protein substrates involved in apoptosis (59,76-78).

8. Perforinopathies

It is recognized that the residual function of the PRF-dependent cytotoxic cells causes transplant rejection of allogenic stem cells, allografts and solid organs. At the opposite pole defects of the cytotoxic path and PRF deficiency (failure to deliver PRF) lead to disorders called perforinopathies including familial haemophagocytic lymphohistiocytosis (FHL), viral infections and the predisposition to develop haemato-oncological diseases (12,79-83). Voskoboinik *et al* have proposed the term of perforinopathies in order to define a spectrum of immune-mediated disease responses associated with monoallelic mutations in genes related to FHL (84).

The complete absence of the PRF function results into FHL, an immunoregulatory disease that appears in childhood and is characterized by uncontrolled activation of CPA and $CD8^+$ T lymphocytes with secondary accumulation of T cells. Recently it was discovered that the partial loss of PRF function is strongly associated with FHL and a series of hematological disorders that appear later in childhood or in adolescence. In addition, PRF functionality is essential for cytotoxic lymphocytes in humans since harmful mutations in PRF1 leads to FHL2 representing 30-60% of FHL cases (59,79,85).

PRF and CD107a tests are more sensitive and have a similar specificity compared with NK cytotoxicity test and would be able to enhance FHL screening (86).

Relative recent studies have revealed that UVB and UVA radiations induce accumulation of granzyme B in human keratinocytes. In addition, granzyme B secondary to UVB radiation mediates cytotoxicity of keratinocytes, while in UVA irradiation it increases the ability of the keratinocyte to degrade matrix extracellular components; these observations could be the basis of photoaging and photocarcinogenicity domain (87,88).

While cancer therapy has begun to use 'suicide genes' to induce cell apoptosis, the role of vaccination with apoptotic cells, either immune stimulatory or immune suppressive is still debated. Recently a new technology called cytolytic DNA technology has been developed, using a vaccine which encodes truncated PRF incorporated in a bicistronic DNA vector that activates dendritic cells thus stimulating the $CD8^+$ T-cell response against HIV and HCV reducing the viral loads similar to traditional vaccines (89-92).

PRF has gained the attention of cardiology researchers, proving to date, that patients with left ventricular dysfunction have PRF-positive infiltration of heart cells and that PRF could be an adverse predictor of long-term mortality in patients with inflammatory cardiomyopathy (93).

9. Conclusions

PRF is a pore-forming protein vital for cytotoxic effector function and has an indispensable role in granzyme-mediated apoptosis. It is responsible for endothelial damage and plays a role in the pathogenesis of numerous inflammatory diseases and targets cell apoptosis.

Over the last few years, the study of serum PRF and its role in inflammatory and neoplastic diseases has captured the attention of the medical world. To date, few studies have reported the correlation between serum or urinary PRF, granzyme and ligand FAS with acute transplant rejection. Further studies are required to clarify the role of PRF as a potential early biomarker with a predictive role in chronic allograft rejection.

Advances in immunosuppressive therapy, in order to maintain kidney transplantation and avoid rejection, have led to decreased rejection rates. However, these agents are not deprived of side effects.

The residual function of PRF-dependent cytotoxic cells causes transplant rejection of allogenic stem cells, the allograft and solid organs. A deep understanding of the role of PRF in inducing allograft rejection is necessary for the development of new targeted post-transplant therapies. Highly specific inhibitors of PRF function, are thus of interest as selective immunosuppressive drugs.

The practical use of PRF expression has already been demonstrated in various medical fields. Demonstrating the utility of PRF as a predictive marker of CAN, such as with PRF inhibitors, would have treatment implications that could mark the beginning of a new era in immunosuppressive therapy.

Acknowledgements

Not applicable.

Funding

The present study was supported by the project ANTREPRENORDOC, in the framework of Human Resources Development Operational Program 2014-2020, financed from the European Social Fund under the contract number 36355/23.05.2019 HRD OP/380/6/13-SMIS Code: 123847.

Availability of data and materials

Not applicable.

Authors' contributions

AMPC conceived, wrote and edited the manuscript. EC reviewed the manuscript for important intellectual content. LAT revised the study before finally approving it for publication. AMPC, EC and LAT confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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