bjh research paper

Effect of mTORC1/mTORC2 inhibition on T cell function: potential role in graft-*versus*-host disease control

Ma Carmen Herrero-Sánchez,[1,2,3] Concepción Rodríguez-Serrano,[1,2,3] Julia Almeida,[2,4] Laura San-Segundo,[2,3] Susana Inogés,[5] Ángel Santos-Briz,[2,6] Jesús García-Briñón,[2,7] Jesús F. SanMiguel,[8] Consuelo Del Cañizo[1,2,3] and Belén Blanco[1,2]

[1]Servicio de Hematología, Hospital Universitario de Salamanca, [2]Instituto de Investigación Biomédica de Salamanca (IBSAL), [3]Centro de Investigación del Cáncer, Universidad de Salamanca, [4]Servicio de Citometría, Centro de Investigación del Cáncer, Universidad de Salamanca, Salamanca, [5]Laboratorio de Inmunoterapia, Clínica Universidad de Navarra, Pamplona, [6]Departamento de Patología, Hospital Universitario de Salamanca, [7]Departamento de Biología Celular y Patología, Facultad de Medicina, Salamanca, and [8]Clínica Universidad de Navarra, Centro de Investigación Médica Aplicada, Instituto de Investigación Sanitaria de Navarra, Pamplona, Spain

Received 16 September 2015; accepted for publication 21 December 2015

Correspondence: Dra. Belén Blanco, Servicio de Hematología, Hospital Universitario de Salamanca, Paseo de San Vicente, 58-182., 37007 Salamanca, Spain.
E-mail: belen_blancod@yahoo.es

Summary

The mechanistic target of rapamycin (mTOR) pathway is crucial for the activation and function of T cells, which play an essential role in the development of graft-*versus*-host disease (GvHD). Despite its partial ability to block mTOR pathway, the mTORC1 inhibitor rapamycin has shown encouraging results in the control of GvHD. Therefore, we considered that simultaneous targeting of both mTORC1 and mTORC2 complexes could exert a more potent inhibition of T cell activation and, thus, could have utility in GvHD control. To assess this assumption, we have used the dual mTORC1/mTORC2 inhibitors CC214-1 and CC214-2. *In vitro* studies confirmed the superior ability of CC214-1 *versus* rapamycin to block mTORC1 and mTORC2 activity and to reduce T cell proliferation. Both drugs induced a similar decrease in Th1/Th2 cytokine secretion, but CC214-1 was more efficient in inhibiting naïve T cell activation and the expression of T-cell activation markers. In addition, CC214-1 induced specific tolerance against alloantigens, while preserving anti-cytomegalovirus response. Finally, in a mouse model of GvHD, the administration of CC214-2 significantly improved mice survival and decreased GvHD-induced damages. In conclusion, the current study shows, for the first time, the immunosuppressive ability of CC214-1 on T lymphocytes and illustrates the role of CC214-2 in the allogeneic transplantation setting as a possible GvHD prophylaxis agent.

Keywords: graft *versus* host disease, T cell, mTOR inhibitors.

Graft-*versus*-host disease (GvHD) remains the leading cause of morbidity and mortality following allogeneic haematopoietic stem-cell transplantation (allo-HSCT). Despite current pharmacological prophylaxis using calcineurin inhibitor-based regimens, grades II–IV acute GvHD and chronic GvHD still develop in up to 50% and 70% of related and unrelated transplantations, respectively (Abouelnasr *et al*, 2013). Therefore, new strategies to prevent and treat GvHD are needed. In recent years, sirolimus has been increasingly used as a GvHD prophylaxis agent, showing encouraging results, especially in combination with tacrolimus (Abouelnasr *et al*, 2013). Perez-Simón *et al* (2013) have reported that the sirolimus/tacrolimus combination achieved a lower incidence of chronic GvHD compared to the standard prophylactic regimen of ciclosporin/mycophenolate mofetil. However, the incidence of both chronic GvHD and acute GvHD remained elevated (Perez-Simón *et al*, 2013).

Sirolimus (rapamycin) is an inhibitor of the kinase mammalian target of rapamycin (mTOR, also termed mechanistic target of rapamycin, MTOR), a critical regulator of cell growth, proliferation, differentiation, metabolism, motility and survival (Wullschleger *et al*, 2006). In T cells, which have an essential role in GvHD development, stimulation of T-cell receptor, costimulatory molecules, such as CD28, and cytokine receptors (e.g. interleukin 2 receptor, IL2R) leads to the activation of mTOR (Waickman & Powell, 2012). Therefore, mTOR inhibition by rapamycin prevents T cell activation and proliferation (Powell *et al*, 1999). However, mTOR exists in two

First published online 23 February 2016
doi: 10.1111/bjh.13984

distinct complexes: mTORC1 and mTORC2. mTORC1 regulates diverse cellular processes, including transcription, ribosome biogenesis and protein synthesis through its targets S6 kinase (S6K) and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1, also termed EIF4EBP1) (Wullschleger *et al*, 2006). mTORC2 phosphorylates targets such as AKT on S473, a critical regulatory site required for maximal AKT kinase activity, and it is thought to mediate cell proliferation and survival (Sparks & Guertin, 2010). Rapamycin inhibits mTORC1, whereas its effects on mTORC2 are variable and generally require prolonged treatment (Sarbassov *et al*, 2006). Thus, rapamycin can allow phosphorylation of mTORC2 targets, which could lead to induction of survival and proliferation. Moreover, the effectiveness of rapamycin may be limited due to the inhibition of mTORC1-dependent negative feedback loops (Wan *et al*, 2007; Rodrik-Outmezguine *et al*, 2011) and to the resistance of 4E-BP1 phosphorylation to long-term rapamycin treatment (Choo *et al*, 2008). All these data show that rapamycin partially blocks mTOR kinase activity. Therefore, we hypothesized that a more potent mTOR inhibitor could more effectively block this pathway in T cells and further reduce the incidence of GvHD.

CC214-1 and CC214-2 (Celgene Corporation, San Diego, CA, USA) are novel molecules with demonstrated antineoplastic activity on several tumour cell types (Gini *et al*, 2013; Mortensen *et al*, 2013; Chen *et al*, 2014b; Demosthenous *et al*, 2015). Both CC214-1 (*in vitro* use) and CC214-2 (*in vivo* use) are highly selective ATP-competitive dual mTOR inhibitors (Gini *et al*, 2013). The ability of CC214-1 and CC214-2 to inhibit both mTORC1 and mTORC2 complexes has been shown on tumour cell lines where RPS6 and 4E-BP1 phosphorylation (markers of mTORC1 activity) and AKT S473 phosphorylation (marker of mTORC2 activity) were inhibited (Gini *et al*, 2013; Mortensen *et al*, 2013; Chen *et al*, 2014b; Demosthenous *et al*, 2015).

The current study evaluated the effect of CC214-1 on T cell activation *in vitro* and the potential utility of CC214-2 in GvHD prophylaxis *in vivo*. We confirmed that CC214-1 reduces cytokine secretion and inhibits, more effectively than rapamycin, T cell proliferation and expression of IL2Rα and cytotoxic molecules. In addition, CC214-1 induces tolerance of alloreactive T cells while preserving immune response against pathogens. Finally, we confirmed in a murine model of GvHD that CC214-2 significantly improves survival of treated mice compared to untreated mice, although, at the tested doses, rapamycin administration provides more benefit than CC214-2. Our results establish the basis for further research on the utility of dual mTOR inhibitors for the control of GvHD.

Methods

Drugs and chemicals

CC214-1 and CC214-2 were kindly provided by Celgene Corporation (San Diego, CA, USA) and rapamycin was purchased from Sigma-Aldrich (St. Louis, MO, USA). For *in vitro* studies, CC214-1 and rapamycin were reconstituted to 10 mmol/l in dimethyl sulphoxide and stored frozen at −20°C until use. For *in vivo* assays, CC214-2 was daily formulated as a suspension in ultrapure water with 0·5% (p/v) carboxymethylcellulose (CMC; Sigma-Aldrich) and 0·25% (v/v) Tween 80 (Sigma-Aldrich) and a stock solution of 10 mg/ml rapamycin was prepared in ethanol, stored at −20°C and diluted in the vehicle solution (0·2% CMC with 0·25% Tween 80 in ultrapure water) before administration.

Cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats of volunteer healthy donors, provided by the Centro de Hemoterapia y Hemodonación de Castilla y León (CHEMCYL). Written informed consents were obtained from all donors in accordance with the Declaration of Helsinki. PBMCs were isolated by density gradient centrifugation using Ficoll-Paque solution (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

For Western blot analysis, cells were allowed to adhere to tissue culture T75 Flasks (Corning, NY, USA) overnight at 37°C. Non-adherent cells were collected to obtain a T-cell enriched PBMC population. For cell cycle, apoptosis and cytokine secretion assays, T cells were purified from PBMCs by immunomagnetic depletion of non-T cells using the Pan T Cell Isolation Kit (Miltenyi Biotec., Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of isolated populations was routinely >95%.

PBMCs or isolated T cells were seeded in well plates (CELLSTAR®, Greiner Bio-One, Frickenhausen, Germany) at a density of 1×10^6 cells/ml in RPMI 1640 medium supplemented with 2 mmol/l L-glutamine, 100 u/ml penicillin, 100 μg/ml streptomycin (all from Gibco-Invitrogen, Paisley, UK) and 10% human AB serum (Sigma-Aldrich) under different culture conditions: in the absence of stimulus or stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (2·5 μg/ml) monoclonal antibodies (mAbs) (BD Biosciences, San Jose, CA, USA).

MTT assays

The inhibitory effect of rapamycin and CC214-1 on stimulated T cells was first analysed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma-Aldrich) colourimetric assay. For that purpose, 10^5 cells/well were stimulated, in triplicate, with anti-CD3/anti-CD28 mAbs in 96-well plates, in the absence or presence of increasing doses (0–10 μmol/l) of rapamycin and CC214-1. After 5 d, 10 μl/well of 0·5 mg/ml MTT were added and, after 2-h incubation at 37°C, formazan crystals were solubilized with 100 μl of 10% sodium dodecyl sulphate (SDS; Merck Millipore, Darmstadt, Alemania) – 0·01 N HCl (Panreac, Barcelona, Spain). Absorbance at 570 nm was measured using a Tecan Ultra Evolution

plate reader (TECAN, Männedorff, Switzerland) with the XFlour4 software (TECAN).

Western blot analysis

T-cell enriched PBMCs (5×10^6)/well were seeded in 6-well plates and cultured in the presence or in the absence of anti-CD3/anti-CD28 mAbs and different concentrations of rapamycin or CC214-1 (0–10 μmol/l). After 48 h, cells were lysed and 30–70 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidenedifluoride membrane (Millipore, Bedford, MA, USA). Membranes were incubated with the following antibodies (from Cell Signaling Thechnology®, Leiden, Netherlands): phospho-AKT (p-AKT) (T308 and S473), total AKT, p-4E-BP1 (T37/46), total-4E-BP1, p-p70S6K (T389), total-p70S6K, p-RPS6 (S235/236), total-RPS6 or caspase 3; antibodies to GAPDH (Cell Signaling Thechnology®) and calnexin (Enzo® Life Science, Plymouth Meeting, PA, USA) were used as protein loading controls. Anti-rabbit conjugated to horseradish peroxidase (GE Healthcare, Buckinghamshire, UK) was used as secondary antibody and bands were visualized using Amersham ECL Western Blotting Detection Reagents (GE Healthcare).

Proliferation assays

T cell proliferation was analysed by flow cytometry. Briefly, PBMCs were stained with PKH-67 green fluorescent dye using PKH67 Green Fluorescent Cell Linker Mini Kit (Sigma-Aldrich) according to manufacturer's instructions. 2×10^5 unstimulated or anti-CD3/anti-CD28 stimulated PKH-stained cells were seeded in 96-well plates with different concentrations of the drugs (0–10 μmol/l). After 5 d cells were collected, stained with 7-amino-actinomycin D (7AAD) and anti-CD3-allophycocyanin (APC) and acquired in a FACSCalibur flow cytometer (BD Biosciences) using the CellQuest software program (BD Biosciences). The percentage of proliferating T cells ($CD3^+$ PKH^{low}) was calculated using the Infinicyt software (Cytognos, Salamanca, Spain).

Cell cycle analysis

After 4 d of culture in the presence of different concentrations of rapamycin or CC214-1 (0–10 μmol/l), unstimulated or stimulated isolated T cells were stained with propidium iodide, using the CycleTEST™ PLUS DNA Reagent Kit (BD Biosciences) according to manufacturer's instructions. A minimum of 20 000 events was acquired on a FACSCalibur flow cytometer using the CellQuest software program. The distribution of cells along the cell-cycle phases was analysed using ModFit LTTM Macintosh program (Verity Software House, Inc., Topsham, ME, USA).

Apoptosis assessment

After 2 d of culture in the presence of different concentrations of the compounds (0–10 μmol/l), unstimulated or stimulated isolated T cells were stained with Annexin V-phycoerythrin (PE) using the PE Annexin V Apoptosis Detection Kit I (BD Pharmingen™, San Diego, CA, USA) according to the manufacturer's instructions. Cells were acquired on a FACSCalibur flow cytometer using the CellQuest software program and the percentage of annexin V-PE T cells was calculated using the Infinicyt software.

Cytokine assays

2×10^5 isolated T cells were cultured in 96-well plates and stimulated with anti-CD3/anti-CD28 mAbs for 48 h in the presence of several concentrations of rapamycin or CC214-1 (0–10 μmol/l). Then, concentration of different cytokines (IL2, IFN-γ, TNF-α, IL4, IL10 and IL6) in culture supernatants was measured using the Human Th1/Th2 Cytokine Cytometric Bead Array (CBA) kit (BD Biosciences), according to manufacturer's instructions. Samples were acquired on a FACSCalibur flow cytometer and analysed using BD CBA software.

Immunophenotypic analysis

2×10^5 unstimulated or anti-CD3/anti-CD28 stimulated PBMCs/well were seeded in 96-well plates and treated with different concentrations of rapamycin or CC214-1 (0–10 μmol/l). After 48 h, cells were stained with the following combination of mAbs: anti-CD45RA-fluorescein isothiocyanate (FITC)/anti-IFN-γ-PE/anti-CD8-peridinin chlorophyll-cyanin 5.5 (PerCP-Cy5.5)/anti-CD25-PE-Cy7/anti-Granzyme B-Alexa Fluor® 647/anti-CD4-APC-Alexa Fluor® 750/anti-CD27-Brillant Violet 421/anti-CD3-Brillant Violet 510. For intracellular staining of IFN-γ and Granzyme B, brefeldin A (10 μg/ml) was added for the last 4 h prior to acquisition and the IntraStain Kit (Dako Cytomation, Glostrup, Denmark) was used. Data acquisition was performed on a FACSCanto flow cytometer (BD) using the FACS DIVA software program (BD Biosciences, San Jose, CA, USA) and analysed using the Infinicyt software.

Enzyme-Linked ImmunoSpot assays

3×10^6 PBMCs from cytomegalovirus (CMV)-positive donors (responder cells) were seeded in 48-well plates and stimulated with irradiated (25 Gy) PBMCs from a second donor (allogeneic target cells) in a 2:1 ratio. Different doses of rapamycin or CC214-1 (0–10 μmol/l) were added. After 96 h, responder cells were collected and cultured, in the absence of drugs, in an IFN-γ Enzyme-Linked ImmunoSpot (ELISPOT) plate (Mabtech, Nacka Strand, Sweeden): (i) in the absence of stimulation (control), (ii) re-stimulated with the same allogeneic cells of the primary culture or (iii) re-stimulated with CMV pp65 recombinant protein (CMV-pp65;

Miltenyi Biotec). After 36 h, ELISPOT was performed, following the manufacturer's instructions. Spots corresponding to IFN-γ secreting cells were quantified using an Immunospot ELISPOT reader (CTL, Aale, Germany) and the Image Acquisition 4.5 and Shortcut to Immunospot 3 software. The frequency of IFN-γ producing cells was determined by subtracting the background spots in the unstimulated (control) wells from the number of spots counted in the corresponding allogeneic or CMV-pp65 re-stimulated wells. These values were normalized with respect to those obtained from the samples pre-stimulated in the absence of drugs (0 μmol/l) and restimulated with allogeneic cells or CMV-pp65, respectively, considered as 100%.

Mouse model of acute GvHD

Female recipient Balb/c (H2d) and male donor C57BL/6J (H2b) mice (12 weeks of age) were purchased from Harlan Laboratory (Carshalton, UK). Animal work was approved by the Ethical Committee of the University of Salamanca.

Balb/c mice received total body irradiation (8·5 Gy in two fractions) from a Cs^{137} source and then an intravenous injection of 10×10^6 C57BL/6 bone marrow cells without (BM group) or with 5×10^6 splenocytes. Mice receiving splenocytes were either untreated (GvHD group) or treated orally with rapamycin 1·5 mg/kg/d (GvHD+rapamycin group) or CC214-2 20 mg/kg/d (GvHD+CC214-2 group), from day +1 to day +60 post-transplantation. Four experiments were performed with at least 2 mice per group. Control mice, which received irradiation without cell support (Total Body Irradiation, TBI group), were also included.

Assessment of GvHD

All Balb/c mice were monitored daily for survival and for the following clinical signs of GvHD: weight loss, posture (hunching), activity, fur texture and skin integrity. Each parameter received a score of 0 (normal), 1 (mild to moderate) or 2 (severe) and a clinical GvHD index was generated subsequently by summation of the five criteria scores (0–10), as previously described (Cooke *et al*, 1996). All moribund mice were humanely killed.

Acute GvHD was also assessed by histopathological analysis of the small and large bowels, liver and skin. For that purpose, one animal of each group was killed in the third week after transplantation and once treatment was completed. Tissues were fixed in 10% neutral-buffered formalin (Sigma-Aldrich), embedded in paraffin, sectioned, slide mounted and stained with haematoxylin and eosin (H&E) (both from Merck KGaA, Darmstadt, Germany). Slides were examined by a pathologist in a blinded fashion. A scoring system was used to determine the degree of GvHD in small and large intestine, skin and liver. Details of the scoring system are summarized in Table I.

Statistical analysis

Statistical analysis was performed using *IBM SPSS Statistics 20* (IBM Corp., Armonk, NY, USA). Differences between different doses of the drugs were analysed by the Kruskal–Wallis multiple comparison *Z* value test. Pairwise comparisons were performed using the Mann–Whitney test with Bonferroni correction. Survival curves were plotted using Kaplan–Meier estimates and a Log-Rank test was used to compare survival rates. Statistical significance in all tests was concluded for values of $P < 0{\cdot}05$.

Results

Rapamycin and CC214-1 exert a dose-dependent inhibitory effect on stimulated T-cells

Initially, the range of concentrations at which the drugs exert an inhibitory effect on human primary T cells was evaluated. For that purpose, colourimetric MTT assays, which provide an estimate of cell viability/proliferation, were performed in samples of anti-CD3/anti-CD28 stimulated PBMCs cultured in the presence of a wide range of concentrations (0–10 μmol/l) of rapamycin and CC214-1. Both inhibitors exerted a dose-dependent effect (Fig S1). At high doses both

Table I. Histological criteria for graft-*versus*-host disease score.

Score	Skin	Liver	Large and small intestine
0	Normal	Normal	Normal
0·5		Focal mild portal lymphoid infiltrate	Occasional or rare necrotic cells in glands or crypts
1	Basal vacuolar change	Widespread mild portal lymphoid infiltrate	Isolated apoptotic epithelial cells, without crypt loss
2	Dyskeratotic cells in the epidermis and/or follicle, dermal lymphocytic infiltrate	Focal bile duct invasion or cellular injury	Individual crypt loss. Regeneration changes
3	Fusion of basilar vacuoles to form clefts and microvesicles	Multiple foci of bile duct injury and regeneration	Contiguous area of multiple crypt loss
4	Separation of epidermis from dermis	Widespread injury and destruction of bile ducts	Extensive crypt dropout with denudation of epithelium

drugs induced similar inhibition, although rapamycin was more efficient than CC214-1 at low concentrations.

Rapamycin and CC214-1 block phosphorylation of mTOR target proteins

The effect of rapamycin and CC214-1 on the phosphorylation of mTOR pathway proteins in stimulated T cells was analysed. Protein phosphorylation increased after stimulation in the absence of drug (Fig 1). Both mTOR inhibitors induced a decrease in the phosphorylation of mTORC1 downstream targets 4E-BP1, p70S6K (S6K) and RPS6, although CC214-1 blocked 4E-BP1 phosphorylation more efficiently than rapamycin. Moreover, there was a reduction not only in 4E-BP1 and RPS6 phosphorylation, but also in their expression. On the other hand, only CC214-1 reduced the phosphorylation of the mTORC2 target AKT S473, whereas rapamycin increased it. Regarding the phosphorylation of AKT on T308, mediated by the mTOR upstream kinase PI3K, none of the drugs induced a decrease; moreover, an increase was observed.

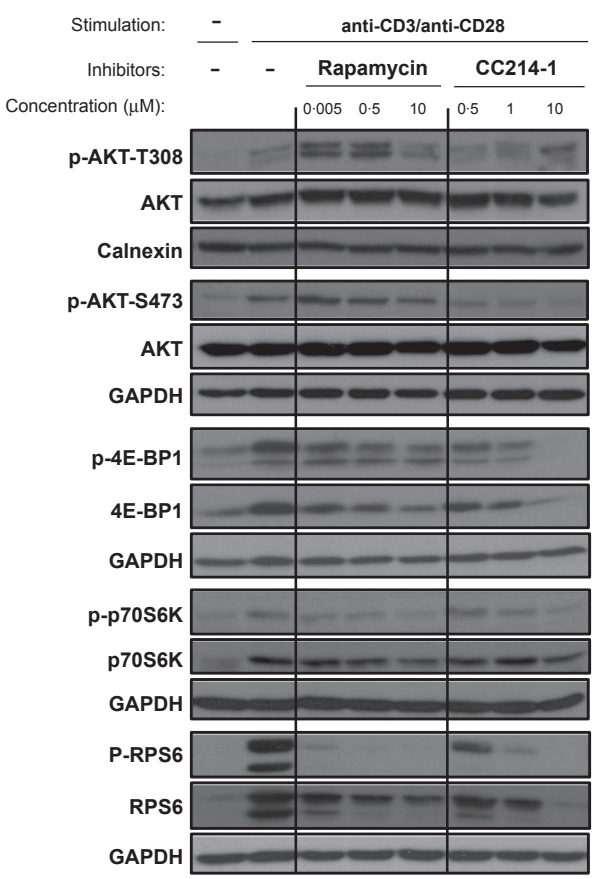

Fig 1. Effect of rapamycin and CC214-1 on PI3K/Akt/mTOR pathway protein phosphorylation. Western blot analysis of phosphorylation and expression of PI3K/AKT/mTOR pathway proteins in stimulated T-cells enriched peripheral blood mononuclear cells for 48 h with anti-CD3 and anti-CD28 monoclonal antibodies, in the presence of different concentrations of rapamycin and CC214-1. At least three independent experiments were performed.

Rapamycin and CC214-1 inhibit T cell proliferation due to cell cycle arrest but not to induction of apoptosis

Rapamycin and CC214-1 was shown to induce a dose-dependent decrease in T-cell proliferation (Fig 2A). Although the effect of rapamycin was more potent at low doses, higher doses of CC214-1 caused greater inhibition.

In order to determine whether the reduction of proliferation was related to cell cycle arrest or apoptosis, both analyses were performed. As shown in Fig 2B, the percentage of cells in synthesis and G2/Mitosis phases significantly decreased in the presence of the drugs. By contrast, none of the mTOR inhibitors induced significant apoptosis in both resting and activated T cells (Fig 3A–B). Accordingly, the amount of cleaved (activated) caspase 3 in stimulated treated cells did not increase with respect to stimulated untreated cells and, moreover, high doses of CC214-1 caused a decrease in activated caspase 3 (Fig 3C).

Rapamycin and CC214-1 diminish Th1/Th2 cytokine production and granzyme B expression

To assess the effect of mTOR inhibitors on the ability of T cells to acquire effector functions, we analysed the capacity of treated T cells to produce activation-related molecules, such as cytokines or the cytotoxic molecule granzyme B, and the expression level of CD25 (also termed IL2Rα, IL2RA).

Cytokine analysis showed that both drugs induced a similar dose-dependent decrease in IL2, IFN-γ, TNF-α, IL4, IL10 and IL6 secretion (Fig 4).

Regarding CD25 expression, rapamycin barely reduced the percentage of $CD25^+$ cells, whereas high doses of CC214-1 caused a more pronounced decrease, significant in $CD8^+$ T lymphocytes (Fig 5). However, rapamycin did decrease CD25 median fluorescence intensity (MFI) (Fig S2). The percentage of granzyme B and IFN-γ expressing cells was diminished by both inhibitors, although this reduction was statistically significant only in the case of CC214-1 (Fig 5).

In addition, T cells were classified into five subsets based on the expression of CD27 and CD45RA: early effector (T_{EE}; $CD45RA^+CD27^{high}$), naïve ($CD45RA^+CD27^+$), central memory (T_{CM}; $CD45RA^-CD27^+$), effector memory (T_{EM}; $CD45RA^-CD27^-$) and effector/TEMRA (effector/terminally differentiated effector memory $CD45RA^+$ cells; $T_{E/T}$; $CD45RA^+CD27^-$) T cells. The percentage and activation state of the different T cell subsets after treatment with both inhibitors were analysed.

After T cell stimulation in the absence of drugs, a population of T_{EE} cells emerged among both $CD4^+$ and $CD8^+$ T cell subsets, but this population decreased with the addition of rapamycin and, especially, of CC214-1. On the contrary, stimulation induced a decrease in the percentage of naïve

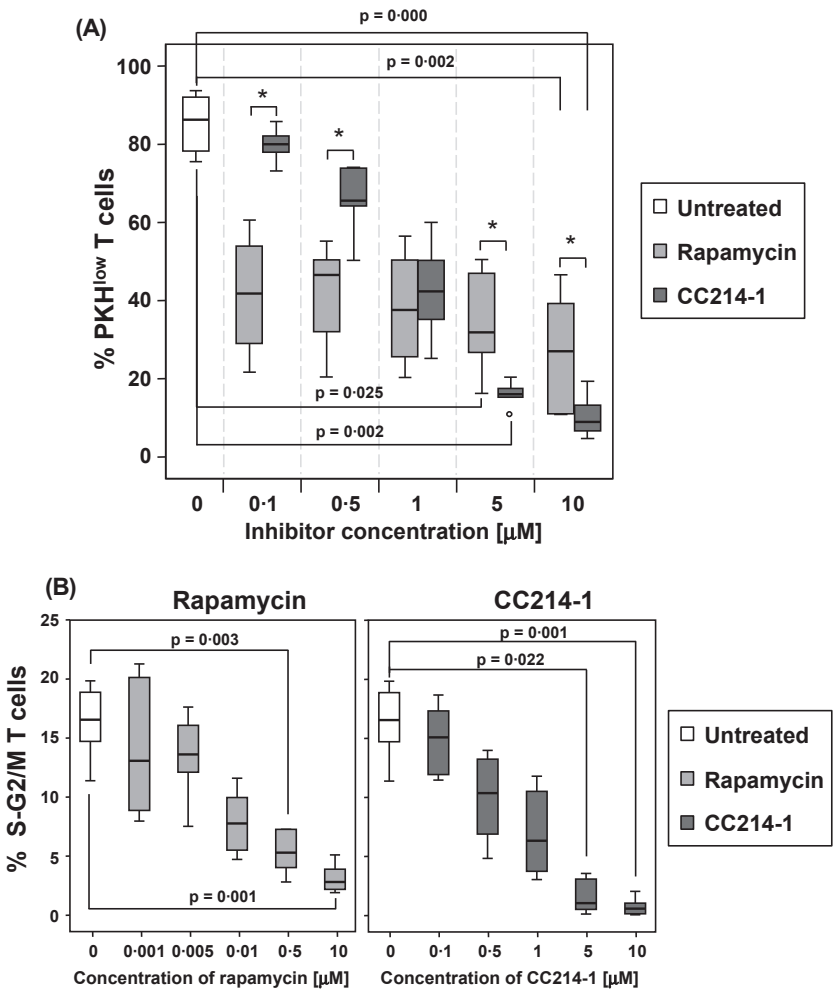

Fig 2. Effect of rapamycin and CC214-1 on T cell proliferation. Peripheral blood mononuclear cells (A) or isolated T cells (B) were stimulated in the presence of different concentrations of rapamycin or CC214-1. (A) Percentage of T cells that had undergone one or more cell divisions ($CD3^+$ PKH^{low}). (B) Percentage of T cells in synthesis and G2/Mitosis (S-G2/M) phases. Six independent experiments were performed. *$P < 0{\cdot}05$.

cells that was strongly reversed by the addition of CC214-1, but poorly modified by rapamycin (Fig 6).

The percentage of T_{CM} cells among $CD4^+$ cells was also reduced as a consequence of stimulation and increased again with the addition of both drugs (data not shown).

Regarding T_{EM} and $T_{E/T}$ cells, the percentage of these subsets changed slightly in the presence of the drugs (data not shown).

The expression of CD25, IFN-γ and granzyme B was also evaluated in the different $CD4^+$ and $CD8^+$ T cell maturation subsets. The effect of the drugs on these subpopulations was similar to that observed on the general $CD4^+$ and $CD8^+$ populations (Figs S3–4). As an exception, the percentage of granzyme B expressing cells among $T_{E/T}$ cells remained high in the presence of the inhibitors. However, MFI analysis showed that the treatment reduced granzyme B expression (Fig S5).

Rapamycin and CC214-1 enable induction of alloreactive T cell anergy while preserving immune response against pathogens

To assess if the inhibitors can induce specific tolerance to alloantigens, PBMCs were stimulated, in the presence of rapamycin or CC214-1, with allogeneic PBMCs and subsequently, in the absence of drugs, with these allogeneic cells or with CMV-pp65 protein.

In samples re-stimulated with allogeneic cells, the percentage of IFN-γ secreting cells diminished among treated cells with respect to untreated control, especially in samples cultured with 10 μmol/l CC214-1. By contrast, in samples re-stimulated with CMV-pp65, IFN-γ secreting cells increased or barely decreased in the presence of both inhibitors compared to the untreated control (Fig 7). These results confirm that both drugs, especially CC214-1, induced tolerization against alloantigens, while preserving anti-CMV response.

Rapamycin and CC214-2 improve survival and clinical state in a murine model of GvHD

Finally, the utility of CC214-2 in GvHD prophylaxis in a mouse model was analysed.

As shown in Fig 8A, CC214-2 treatment significantly increased survival compared to untreated mice ($P = 0{\cdot}022$). However, the best results were achieved with rapamycin (100% survival at 60 d post-transplantation).

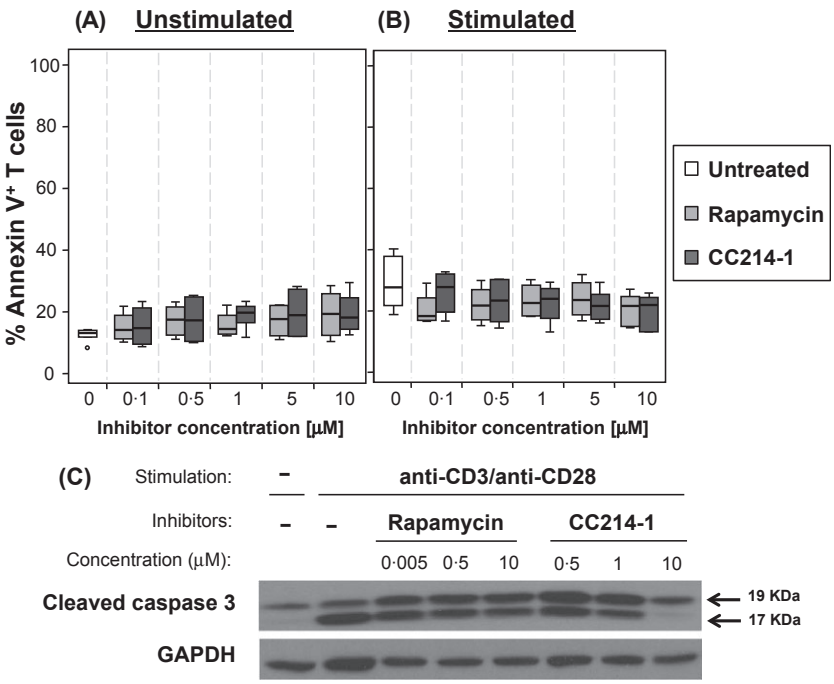

Fig 3. Effect of rapamycin and CC214-1 on induction of T cells apoptosis. Percentage of annexin V^+ T cells among unstimulated (A) or stimulated (B) isolated T cells cultured in the presence of different concentrations of rapamycin or CC214-1. Data pooled from four independent experiments. (C) Western blot analysis of cleaved caspase 3 in T-cell enriched peripheral blood mononuclear cells. Results representative from three independent experiments.

CC214-2 treatment did not reduce the weight loss suffered by mice as a consequence of transplantation, except in mice surviving beyond day 60 post-transplantation (Fig 8B–C). By contrast, weight was greatly improved in rapamycin-treated mice (Fig 8B). Similarly, CC214-2 slightly improved GvHD-related signs, except in those mice surviving beyond day 60 post-transplantation (Fig 8D–E), in which GvHD signs were substantially milder than in untreated mice. Once more, rapamycin treatment greatly ameliorated GvHD-related signs (Fig 8D).

To evaluate GvHD target organ damage, histopathological analysis of skin, liver and intestines were performed in the third week post-transplantation and after the treatment was completed. Only untreated mice showed significant cutaneous GvHD signs on the skin (Fig 9A). Large bowel samples also demonstrated a protective effect both of CC214-2 and rapamycin on colon GvHD development (Fig 9B). However, both untreated and treated mice showed lymphocytic infiltration in periportal areas, although it was less pronounced in mice receiving rapamycin (Fig 9C). Regarding the small intestine, neither untreated nor treated mice showed significant GvHD pathology (data not shown).

Discussion

Despite the many advances that have reduced the incidence of severe GvHD and its associated mortality, GvHD continues to be a major limitation to successful allo-HSCT. In recent years, the clinical use of rapamycin has shown promising results but the high relapse rate and incidence of GvHD remain challenging (Abouelnasr *et al*, 2013; Perez-Simon *et al*, 2013). The immunosuppressive activity of rapamycin relies on its ability to target the mTOR pathway, which is critical for the activation and function of T lymphocytes, key players in GvHD development. As rapamycin incompletely blocks the mTOR pathway (Wan *et al*, 2007; Choo *et al*, 2008; Julien *et al*, 2010; Rodrik-Outmezguine *et al*, 2011), we hypothesized that a more potent mTOR inhibitor could further reduce alloreactive T cell activation and, therefore, decrease the incidence of GvHD. Thus, the aim of the present work was to evaluate, in comparison with rapamycin, the efficacy of the dual mTORC1/mTORC2 inhibitors CC214-1 and CC214-2 in human primary T cell inhibition and in a murine model of GvHD, respectively.

With this aim, the ability of CC214-1 to inhibit mTORC1 and mTORC2 activity in human T cells was first assessed. We confirmed, as shown in other cell types (Gini *et al*, 2013; Chen *et al*, 2014b; Demosthenous *et al*, 2015), that CC214-1 effectively blocked the phosphorylation of downstream targets of both mTORC1 (S6K, RPS6 and 4E-BP1) and mTORC2 (AKT S473). Regarding rapamycin, our results are in accordance with previous reports showing that, while rapamycin inhibits phosphorylation of S6K and RPS6, it has a modest effect on 4E-BP1 phosphorylation (Choo *et al*, 2008; Feldman *et al*, 2009; Thoreen *et al*, 2009). Other authors have found similar differences in mTOR downstream target phosphorylation between rapamycin and dual mTORC1/mTORC2 inhibitors (Feldman *et al*, 2009; Thoreen *et al*, 2009).

On the other hand, not only phosphorylation but also expression of both RPS6 and 4E-BP1 was reduced upon mTOR inhibition. It has been shown that the synthesis of ribosomal proteins, such as RPS6, is tightly linked to cell cycle progression and remains low during the G_0 phase (Nos-

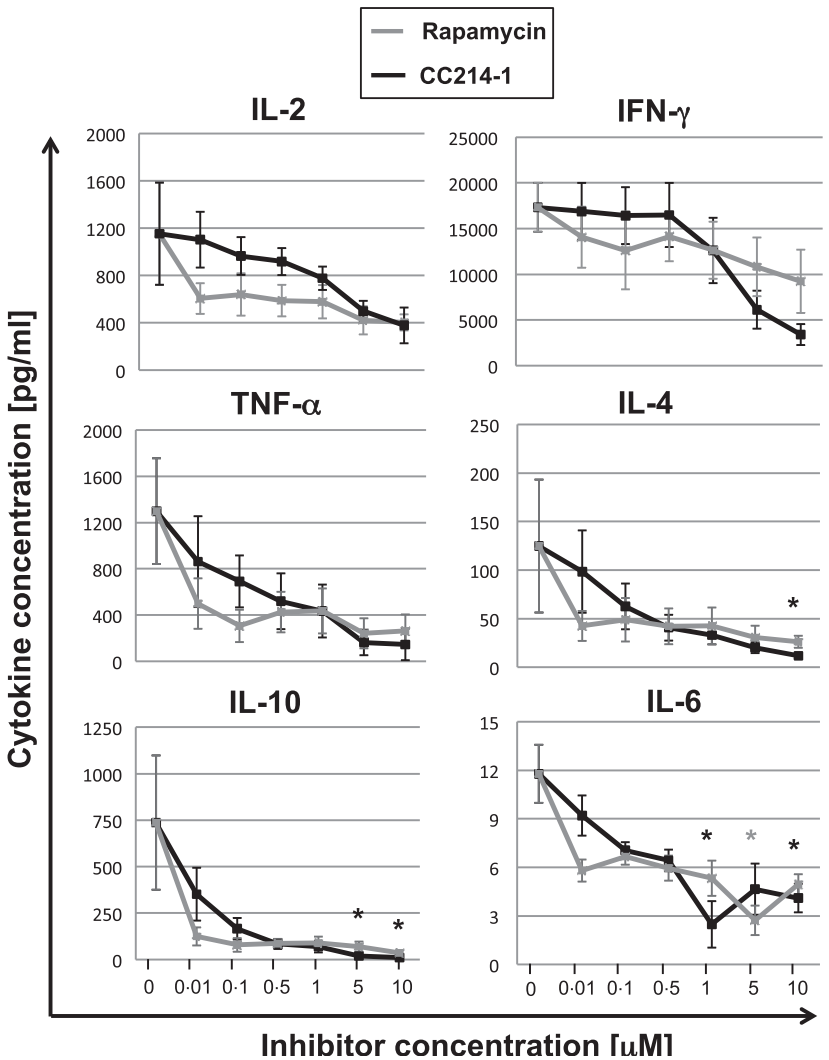

Fig 4. Effect of rapamycin and CC214-1 on T cell cytokine secretion. Concentration of IL2, IFN-γ, TNF-α, IL4, IL10, IL6 in the culture supernatant of isolated T cells stimulated in the presence of different concentrations of rapamycin or CC214-1. Data represent the mean ± SEM of at least three independent experiments. *$P < 0{\cdot}05$ with respect to stimulated untreated samples (0 μmol/l).

rati *et al*, 2014), as observed in resting T cells. Thus, it is probable that the antiproliferative effect induced by mTOR inhibitors impairs the expression of RPS6. Regarding 4E-BP1, its expression is negatively regulated by ERK pathway. It has been shown that mTORC1 inhibition relieves the break that S6K exerts on PI3K-RAS signalling (Carracedo *et al*, 2008), increasing RAS/RAF/MEK/ERK pathway activity. Therefore, the reduced 4E-BP1 expression caused by rapamycin and CC214-1 could be due to an increase in the activity of ERK as a consequence of mTORC1 inhibition.

With respect to mTORC2 activity, rapamycin did not reduce phosphorylation of its target AKT S473. Rapamycin does not bind mTOR when forming the mTORC2 complex and thus can allow phosphorylation of targets such as AKT S473 (Sarbassov *et al*, 2005). Although high doses or prolonged exposure allow rapamycin binding to free mTOR and prevent *de novo* mTORC2 assembly and activity (Sarbassov *et al*, 2006; Tomasoni *et al*, 2011), only a few uninhibited mTORC2 complexes could be sufficient to phosphorylate AKT (Sparks & Guertin, 2010). Furthermore, as previously shown (Sun *et al*, 2005), we have observed that rapamycin is able to increase p-AKT S473 levels. In this sense, it has been reported that S6K, downstream of mTORC1, directly phosphorylates mTORC2, reducing its ability to phosphorylate Akt S473 (Julien *et al*, 2010). Thus, inhibition of mTORC1 relieves this negative feedback of S6K on mTORC2, presumably inducing an increase of AKT S473 phosphorylation. In addition, mechanisms of feedback inhibition exerted by mTORC1 and downstream targets on PI3K signalling have been described (Wan *et al*, 2007) and recent evidence suggests that PI3K signalling directly mediates mTORC2 activity (Gan *et al*, 2011). Thus, the loss of the inhibitory feedback on PI3K upon rapamycin inhibition of mTORC1 could contribute to an increased activation of mTORC2 and the subsequent AKT S473 phosphorylation. Moreover, the inhibition of mTORC1 exerted by both rapamycin and CC214-1 may result in an enhanced PI3K signalling, reflected in increased AKT T308 phosphorylation (Rodrik-Outmezguine *et al*, 2011), as observed at some drug concentrations.

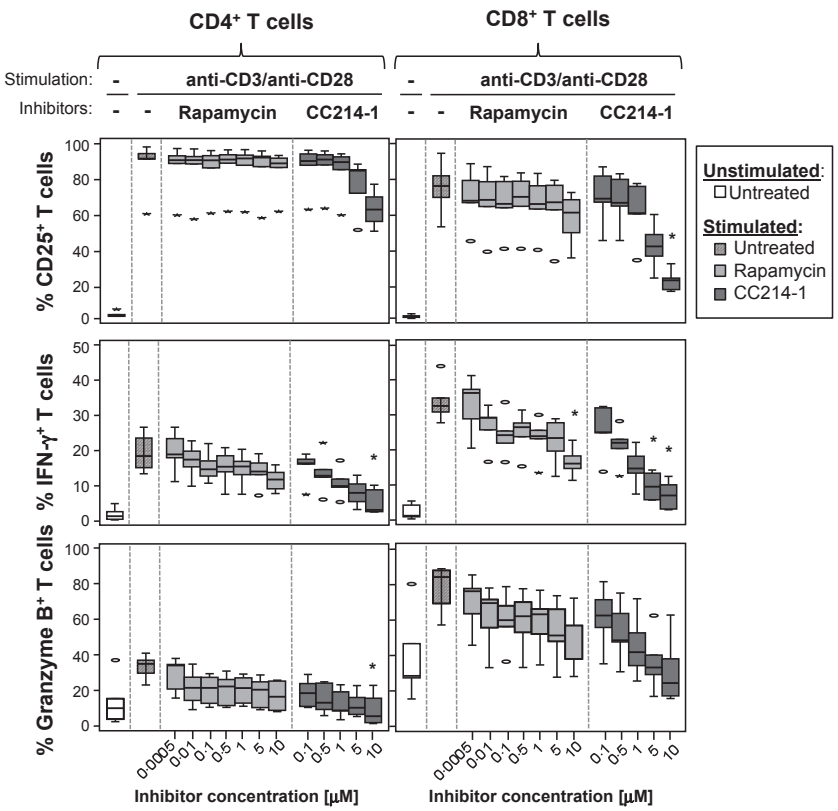

Fig 5. Effect of rapamycin and CC214-1 on expression of T cell activation markers: Percentage of $CD25^+$, IFN-γ^+ and granzyme B^+ cells among $CD4^+$ and $CD8^+$ T cells unstimulated or stimulated in the presence of different concentrations of CC214-1 and rapamycin. Data pooled from five independent experiments. $^*P < 0{\cdot}05$ with respect to stimulated untreated samples (0 μmol/l).

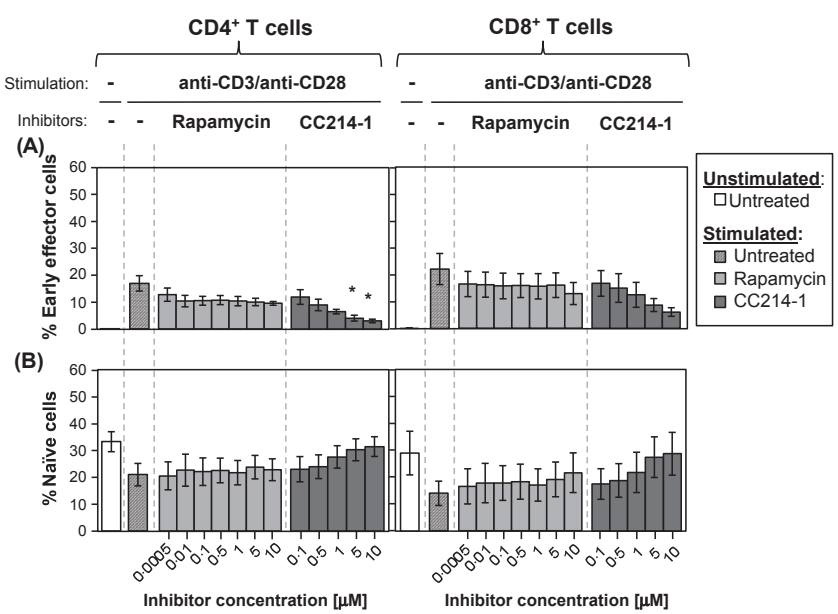

Fig 6. Effect of rapamycin and CC214-1 on the percentage and phenotype of T cell maturation subsets. Percentage of (A) early effector and (B) naïve cells among $CD4^+$ and $CD8^+$ cells unstimulated or stimulated in the presence of different concentrations of rapamycin or CC214-1. Mean ± SEM of five different experiments. $^*P < 0{\cdot}05$ with respect to stimulated untreated samples (0 μmol/l).

Collectively, protein phosphorylation analysis confirms that rapamycin exerts a weaker inhibition on mTOR signalling than CC214-1. Accordingly, although the inhibitory effect of rapamycin on T cell proliferation was evident at low doses, CC214-1 was more effective at high concentrations. Probably, rapamycin-resistant phosphorylation of 4E-BP1 and mTORC2 targets, which drive the synthesis and activity of cell cycle-related proteins (Manning & Cantley, 2007; Barnhart *et al*, 2008), help to maintain higher proliferation than CC214-1 treatment.

On the other hand, mTOR inhibitors did not induce apoptosis in T cells. Moreover, the highest dose of CC214-1 decreased the amount of cleaved caspase 3 in stimulated cells compared to untreated cells. Given that cleavage of caspase-3

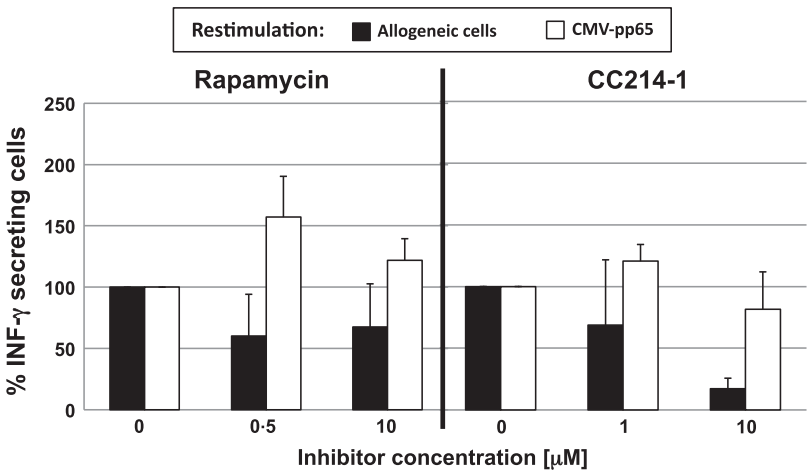

Fig 7. Effect of rapamycin and CC214-1 on T cell tolerization: Percentage of IFN-γ secreting cells among lymphocytes pre-stimulated with allogeneic cells in the presence of different doses of rapamycin or CC214-1 and re-stimulated, in the absence of drugs, with the same allogeneic cells or with CMV-pp65. Every value was normalized to the number of IFN-γ secreting cells pre-stimulated in the absence of drugs (0 mmol/l) and subjected to the corresponding kind of restimulation. Results are means ± SEM of three independent experiments.

occurs in activated T lymphocytes in the absence of apoptosis, as a part of the T-cell activation process (Alam *et al*, 1999), inhibition of T cell activation could be inducing this decrease in caspase-3 cleavage.

However, as AKT has a critical role in promoting cell survival (Manning & Cantley, 2007), we would expect that CC214-1 induced the increase in apoptosis by inhibiting AKT S473 phosphorylation. It is possible that phosphorylation on T308 is sufficient to maintain AKT-induced survival. The observation that rapamycin does not induce apoptosis is less surprising, as it allows AKT phosphorylation. In fact, although rapamycin has been shown to induce T cell apoptosis in some conditions (Li *et al*, 1999), resistance of rapamycin-treated T lymphocytes to cell death has also been reported (Slavik *et al*, 2004; Mariotti *et al*, 2008; Amarnath *et al*, 2010), associated to the regulation of expression of anti- and pro-apoptotic proteins (Mariotti *et al*, 2008; Amarnath *et al*, 2010).

Regarding expression of T cell activation markers, CC214-1 induced a decrease in the percentage of $CD25^+$ T cells, whereas rapamycin hardly reduced it. In accordance, it has been described that in T cells the activity of the principal activator of CD25 transcription, NF-κB (Algarte *et al*, 1995), is dependent on protein kinase C (PKC), downstream of mTORC2 (Sparks & Guertin, 2010). Both drugs decreased the expression of granzyme B and IFN-γ, which is dependent on the mTORC1/mTORC2 regulated transcription factors T-bet and EOMES (Pearce *et al*, 2003; Glimcher *et al*, 2004; Rao *et al*, 2010). in line with this, it has been previously shown that rapamycin inhibits IFN-γ and granzyme B expression (Blazar *et al*, 1998; Nikolaeva *et al*, 2006; Rao *et al*, 2010; Tomasoni *et al*, 2011), and, therefore, cytotoxic activity (Makrigiannis & Hoskin, 1997; Blazar *et al*, 1998). Here, we have shown that a more effective inhibition of mTOR signalling translates into lower expression of these molecules.

Cytokine secretion assays confirmed that IFN-γ production was more affected by CC214-1 than by rapamycin. However, the rest of cytokines tested similarly reduced with high doses of both inhibitors. Rapamycin-induced inhibition of Th1 and Th2 cytokine secretion have been previously described (Chen *et al*, 2014a) and is logical, as mTOR regulates the activity of T-bet and GATA3, the transcription factors that drive Th1 and Th2 differentiation, respectively (Lee *et al*, 2010; Delgoffe *et al*, 2011; Tomasoni *et al*, 2011).

T cells can be classified into different maturation stages, according to the expression pattern of CD27 and CD45RA: T_{EE}, naïve, T_{CM}, T_{EM} and $T_{E/T}$ (Tomiyama *et al*, 2004). The contribution of these subsets of donor T cells to the pathogenesis of GvHD has been studied, highlighting the role of the naïve T cell in GvHD induction (Juchem *et al*, 2011), due to their wide TCR repertoire and to their ability to migrate to lymphatic nodes, proliferate and differentiate to effector and memory T cells upon stimulation (Juchem *et al*, 2011).

Our results showed that CC214-1 inhibited naïve T cell activation more effectively than rapamycin, as indicated by the reduction in the percentage of T_{EE} cells, which arise from naïve T cells after stimulation (Obar & Lefrancois, 2010). Furthermore, decreased CD25, IFN-γ and granzyme B expression in both naïve and T_{EE} cells was more pronounced in CC214-1 treated cells.

GvHD prophylactic strategies are associated with a high risk of infections by pathogens, such as CMV (van Burik *et al*, 2007). Thus, a good GvHD prophylactic strategy should, ideally, induce tolerance of alloreactive T cells while maintaining an adequate immune response against pathogens. Given that rapamycin administration after allo-HSCT has been associated with a lower incidence of GvHD (Abouelnasr *et al*, 2013) and a reduced CMV reactivation (Marty *et al*, 2007), we hypothesized that administration of mTOR kinase inhibitors could generate tolerance of alloreactive T cells but preserve lymphocyte reactivity against other antigens. ELISPOT assays confirmed that treatment with mTOR inhibitors during allogeneic PBMC stimulation decreased the capacity to produce IFN-γ upon allogeneic

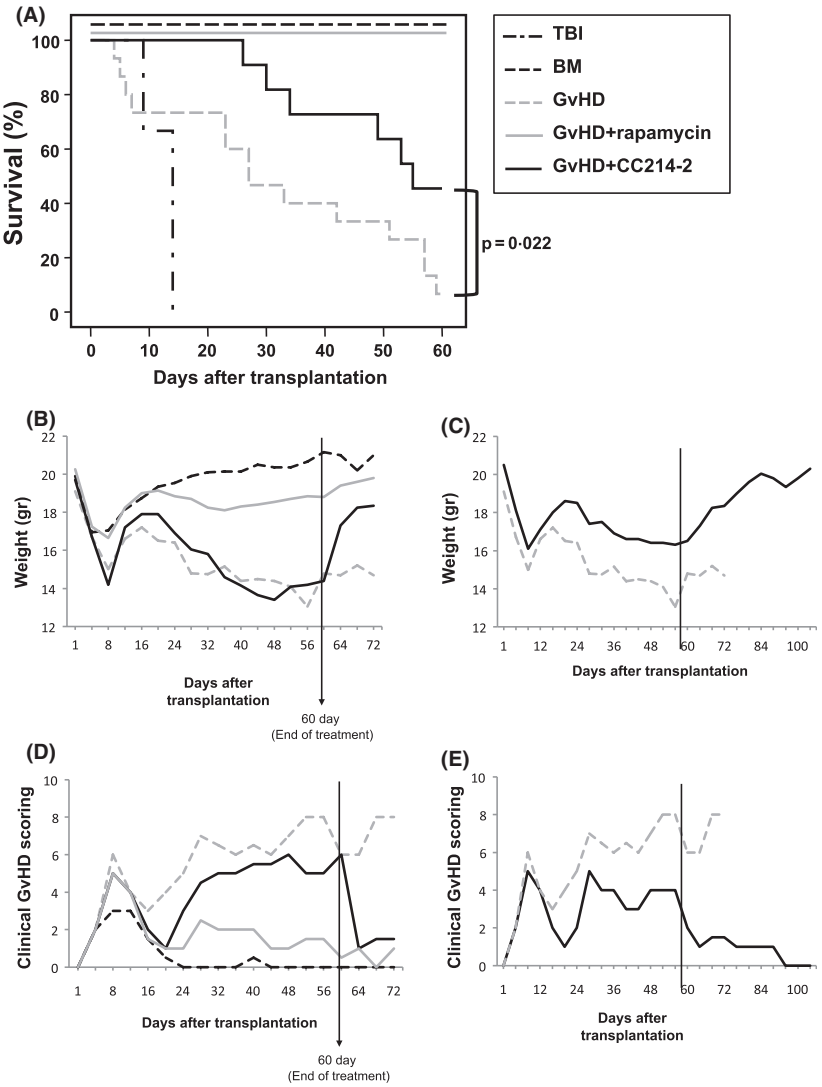

Fig 8. Survival and GvHD signs of the different experimental groups in a GvHD murine model. (A) Kaplan–Meier curve representing overall survival of the different experimental groups: Total body irradiation (TBI; $n = 4$), bone marrow (BM; $n = 8$), graft-*versus* host disease (GvHD; $n = 15$), GvHD+rapamycin ($n = 8$) and GvHD+CC214-2 ($n = 11$). (B) Evolution of weight loss of transplanted mice (median weight in g). (C) Median weight of GvHD group and of those mice from GvHD+CC214-2 group that survived more than 60 d post-transplantation. (D) GvHD score of transplanted mice (median). (E) GvHD score of GvHD group and of those mice from GvHD+CC214-2 group that survived more than 60 d post-transplantation.

restimulation, but maintained, and even increased, the ability to respond to CMV stimulation. This result could be related to the observed protective effect of sirolimus-based GvHD prophylaxis regimens on CMV reactivation (Marty *et al*, 2007). In addition, despite its immunosuppressive properties, rapamycin exhibits immunostimulatory effects on virus and vaccine-induced memory $CD8^+$ T-cell differentiation and activity (Araki *et al*, 2009). In our short-term cultures, differentiation of naïve/early effectors to memory T cells is improbable. Thus, the intensification of pre-existing CMV-specific memory T cell response as a consequence of the incubation with the inhibitors should be presumably occurring. Strikingly, in flow cytometric assays, the treatment with mTOR inhibitors did not enhance, but diminished memory T cell activation, including IFN-γ production. It should be noted that polyclonal stimulation with anti-CD3/anti-CD28 antibodies was performed in these studies, so all the cells were stimulated at the time of drug administration. However, in ELISPOT assays, only allogeneic cells were stimulated in the presence of the inhibitors. It is probable that mTOR inhibition exerts an immunosuppressive effect only on cells that are stimulated in the presence of the drugs, but an immunostimulatory effect on unstimulated T cells. In fact, in the *in vivo* studies, where rapamycin improved the generation of memory $CD8^+$ T cells, rapamycin was administrated prior to vaccination/infection or several days later (Araki *et al*, 2009; Turner *et al*, 2011).

Therefore, we postulate that the administration of mTOR inhibitors at the time of stimulation has an immunosuppressive effect, whereas the administration pre- or post-stimulation strengthens memory immune response.

In vitro results, showing that CC214-1 inhibits T cell activation more efficiently than rapamycin, encouraged us to evaluate the potential utility of CC214-2 in GvHD prophylaxis. Administration of CC214-2 significantly improved mice survival and ameliorated GvHD signs. Moreover, histopathological analysis showed limited GvHD-associated damage in target organs. However, rapamycin provided better results. It

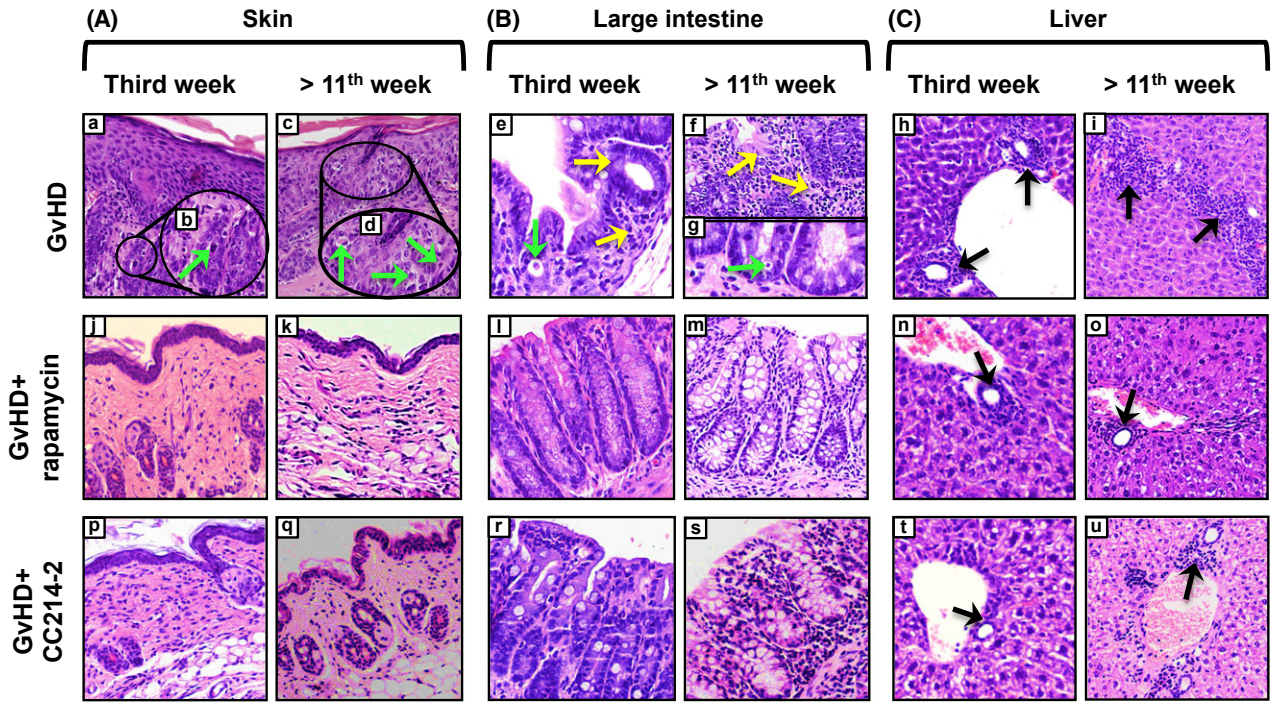

Fig 9. Histopathological analysis. Samples from skin (A), large bowel (B) and liver (C) were obtained from graft-*versus* host disease (GvHD) mice, untreated or treated with rapamycin or CC214-2, in the 3rd week after transplantation and once treatment was completed (beyond 11th week after transplantation). Apoptotic bodies (green arrows), loss of crypts and caliciform cells (yellow arrows) in large bowel and lymphocytic infiltration in periportal areas (black arrows) in liver are indicated. Original magnification: panels b, d, e, g and l: ×400; all other panels: ×200.

is probable that the more potent blockade of mTOR exerted by CC214-2 could be determining a toxic effect, as the mTOR pathway is essential for many vital cellular processes. In fact, treatment with mTOR inhibitors has been associated with certain adverse effects, such as metabolic disorders, defects in haematopoiesis and damage of several organs (Kaplan *et al*, 2014). Moreover, inhibition of the PI3K/mTOR pathway in mice has been reported to induce weight loss, associated with a lower food intake (Wang *et al*, 2014). Further studies evaluating different doses/schedules of administration are warranted in order to reduce CC214-2 toxicity.

On the other hand, dual mTOR inhibitors have been reported to show a higher antitumoural effect than rapamycin (Zhou & Huang, 2012), which could represent an advantage for reducing the incidence of relapse after transplantation. Moreover, as mTOR inhibition has been shown to have a beneficial effect in the prevention and treatment of post-transplant lymphoproliferative disorder (Cullis *et al*, 2006; Pascual, 2007; Ferreira *et al*, 2015), the blockade of both mTORC complexes could provide superior anti-proliferative benefit compared to mTORC1 targeting, as suggested by the results of Furukawa *et al* (2013). Likewise, we should consider the effect of CC214 compounds on the Treg population, whose potential role in controlling GvHD has been described (Hess, 2006). In this regard, several studies have suggested that the generation/expansion of Tregs is more effective when both mTORC complexes are inhibited (Lee *et al*, 2010; Delgoffe *et al*, 2011).

In summary, we have shown for the first time that CC214-1 exerts an immunosuppressive effect on stimulated T cells and induces tolerance in alloreactive T cells while preserving immune response against pathogens. In addition, we have demonstrated that CC214-2 reduces GvHD mortality in an animal model. These results support the potential utility of dual mTORC1/mTORC2 inhibitors in the context of allo-HSCT.

Acknowledgements

The authors would like to thank Centro de Hemoterapia y Hemodonación de Castilla y León (CHEMCYL, Valladolid) for providing buffy coats; co-workers at the Cell Therapy Laboratory (Hospital Universitario de Salamanca) and at Laboratory 12 (Centro de Investigación del Cáncer, Salamanca) for their kind assistance; Javier Borrajo (Departamento de Física, Ingeniería y Radiología Médica, USAL) for mouse irradiation. MCH-S was supported by a grant from the Fundación Española de Hematología y Hemoterapia. BB was supported by a grant from the Fundación Científica de la Asociación Española Contra el Cáncer. This project has been funded with a grant from the Gerencia Regional de Salud de Castilla y León GRS 876/A/13.

Author contributions

MCH-S performed *in vitro* and *in vivo* experiments, analysed data and wrote the manuscript; CR-S performed *in vitro* experiments and data analysis; JA contributed to flow cytometry research; LS-S contributed to *in vivo* experiments; SI contributed to ELISPOT assays; AS-B analysed histopathology samples; JG-B contributed to tissue sample collection; JFSM conceived the research project; CdC supervised the research project and critically reviewed the manuscript; BB designed the research project, performed *in vitro* and *in vivo* experiments, analysed data and critically reviewed the manuscript.

Conflicts of interest

None declared.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Assessment of the inhibitory effect of rapamycin and CC214-1 in stimulated T cells.

Fig S2. Effect of rapamycin and CC214-1 on CD25 expression by T cells.

Fig S3. Effect of rapamycin and CC214-1 on the phenotype of the different $CD4^+$ T cell maturation subsets.

Fig S4. Effect of rapamycin and CC214-1 on the phenotype of the different $CD8^+$ T cell maturation subsets.

Fig S5. Effect of rapamycin and CC214-1 on granzyme B expression by effector T cells.

References

Abouelnasr, A., Roy, J., Cohen, S., Kiss, T. & Lachance, S. (2013) Defining the role of sirolimus in the management of graft-versus-host disease: from prophylaxis to treatment. *Biology of Blood and Marrow Transplantation: Journal of the American Society for Blood and Marrow Transplantation*, **19**, 12–21.

Alam, A., Cohen, L.Y., Aouad, S. & Sekaly, R.P. (1999) Early activation of caspases during T lymphocyte stimulation results in selective substrate cleavage in nonapoptotic cells. *The Journal of Experimental Medicine*, **190**, 1879–1890.

Algarte, M., Lecine, P., Costello, R., Plet, A., Olive, D. & Imbert, J. (1995) In vivo regulation of interleukin-2 receptor alpha gene transcription by the coordinated binding of constitutive and inducible factors in human primary T cells. *The EMBO Journal*, **14**, 5060–5072.

Amarnath, S., Flomerfelt, F.A., Costanzo, C.M., Foley, J.E., Mariotti, J., Konecki, D.M., Gangopadhyay, A., Eckhaus, M., Wong, S., Levine, B.L., June, C.H. & Fowler, D.H. (2010) Rapamycin generates anti-apoptotic human Th1/Tc1 cells via autophagy for induction of xenogeneic GVHD. *Autophagy*, **6**, 523–541.

Araki, K., Turner, A.P., Shaffer, V.O., Gangappa, S., Keller, S.A., Bachmann, M.F., Larsen, C.P. & Ahmed, R. (2009) mTOR regulates memory CD8 T-cell differentiation. *Nature*, **460**, 108–112.

Barnhart, B.C., Lam, J.C., Young, R.M., Houghton, P.J., Keith, B. & Simon, M.C. (2008) Effects of 4E-BP1 expression on hypoxic cell cycle inhibition and tumor cell proliferation and survival. *Cancer Biology & Therapy*, **7**, 1441–1449.

Blazar, B.R., Taylor, P.A., Panoskaltsis-Mortari, A. & Vallera, D.A. (1998) Rapamycin inhibits the generation of graft-versus-host disease- and graft-versus-leukemia-causing T cells by interfering with the production of Th1 or Th1 cytotoxic cytokines. *The Journal of Immunology*, **160**, 5355–5365.

van Burik, J.A., Carter, S.L., Freifeld, A.G., High, K.P., Godder, K.T., Papanicolaou, G.A., Mendizabal, A.M., Wagner, J.E., Yanovich, S. & Kernan, N.A. (2007) Higher risk of cytomegalovirus and aspergillus infections in recipients of T cell-depleted unrelated bone marrow: analysis of infectious complications in patients treated with T cell depletion versus immunosuppressive therapy to prevent graft-versus-host disease. *Biology of Blood and Marrow Transplantation: Journal of the American Society for Blood and Marrow Transplantation*, **13**, 1487–1498.

Carracedo, A., Ma, L., Teruya-Feldstein, J., Rojo, F., Salmena, L., Alimonti, A., Egia, A., Sasaki, A.T., Thomas, G., Kozma, S.C., Papa, A., Nardella, C., Cantley, L.C., Baselga, J. & Pandolfi, P.P. (2008) Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. *The Journal of Clinical Investigation*, **118**, 3065–3074.

Chen, S., Liu, D., Wu, J., Xu, B., Lu, K., Zhu, W. & Chen, M. (2014a) Effect of inhibiting the signal of mammalian target of rapamycin on memory T cells. *Transplantation Proceedings*, **46**, 1642–1648.

Chen, X., Diaz-Rodriguez, E., Ocio, E.M., Paiva, B., Mortensen, D.S., Lopez-Girona, A., Chopra, R., Miguel, J.S. & Pandiella, A. (2014b) Genetic and pharmacologic evidence that mTOR targeting outweighs mTORC1 inhibition as an anti-myeloma strategy. *Molecular Cancer Therapeutics*, **13**, 504–516.

Choo, A.Y., Yoon, S.O., Kim, S.G., Roux, P.P. & Blenis, J. (2008) Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 17414–17419.

Cooke, K.R., Kobzik, L., Martin, T.R., Brewer, J., Delmonte, Jr, J., Crawford, J.M. & Ferrara, J.L. (1996) An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation: I. The roles of minor H antigens and endotoxin. *Blood*, **88**, 3230–3239.

Cullis, B., D'Souza, R., McCullagh, P., Harries, S., Nicholls, A., Lee, R. & Bingham, C. (2006) Sirolimus-induced remission of posttransplantation lymphoproliferative disorder. *American Journal of Kidney Diseases: The Official Journal of the National Kidney Foundation*, **47**, e67–e72.

Delgoffe, G.M., Pollizzi, K.N., Waickman, A.T., Heikamp, E., Meyers, D.J., Horton, M.R., Xiao, B., Worley, P.F. & Powell, J.D. (2011) The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2. *Nature Immunology*, **12**, 295–303.

Demosthenous, C., Han, J.J., Stenson, M.J., Maurer, M.J., Wellik, L.E., Link, B., Hege, K., Dogan, A., Sotomayor, E., Witzig, T. & Gupta, M. (2015) Translation initiation complex eIF4F is a therapeutic target for dual mTOR kinase inhibitors in non-Hodgkin lymphoma. *Oncotarget*, **6**, 9488–9501.

Feldman, M.E., Apsel, B., Uotila, A., Loewith, R., Knight, Z.A., Ruggero, D. & Shokat, K.M. (2009) Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. *PLoS Biology*, **7**, e38.

Ferreira, H., Bustorff, M., Santos, J., Ferreira, I., Sampaio, S., Salome, I., Bastos, J., Bergantim, R., Principe, F. & Pestana, M. (2015) Post-transplant lymphoproliferative disorder: a single-center experience. *Transplantation Proceedings*, **47**, 981–984.

Furukawa, S., Wei, L., Krams, S.M., Esquivel, C.O. & Martinez, O.M. (2013) PI3Kdelta inhibition augments the efficacy of rapamycin in suppressing proliferation of Epstein–Barr virus (EBV)+ B cell lymphomas. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons*, **13**, 2035–2043.

Gan, X., Wang, J., Su, B. & Wu, D. (2011) Evidence for direct activation of mTORC2 kinase activity by phosphatidylinositol 3,4,5-trispho-

sphate. *The Journal of Biological Chemistry*, **286**, 10998–11002.

Gini, B., Zanca, C., Guo, D., Matsutani, T., Masui, K., Ikegami, S., Yang, H., Nathanson, D., Villa, G.R., Shackelford, D., Zhu, S., Tanaka, K., Babic, I., Akhavan, D., Lin, K., Assuncao, A., Gu, Y., Bonetti, B., Mortensen, D.S., Xu, S., Raymon, H.K., Cavenee, W.K., Furnari, F.B., James, C.D., Kroemer, G., Heath, J.R., Hege, K., Chopra, R., Cloughesy, T.F. & Mischel, P.S. (2013) The mTOR kinase inhibitors, CC214-1 and CC214-2, preferentially block the growth of EGFRvIII-activated glioblastomas. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*, **19**, 5722–5732.

Glimcher, L.H., Townsend, M.J., Sullivan, B.M. & Lord, G.M. (2004) Recent developments in the transcriptional regulation of cytolytic effector cells. *Nature Reviews Immunology*, **4**, 900–911.

Hess, A.D. (2006) Modulation of graft-versus-host disease: role of regulatory T lymphocytes. *Biology of Blood and Marrow Transplantation: Journal of the American Society for Blood and Marrow Transplantation*, **12**, 13–21.

Juchem, K.W., Anderson, B.E., Zhang, C., McNiff, J.M., Demetris, A.J., Farber, D.L., Caton, A.J., Shlomchik, W.D. & Shlomchik, M.J. (2011) A repertoire-independent and cell-intrinsic defect in murine GVHD induction by effector memory T cells. *Blood*, **118**, 6209–6219.

Julien, L.A., Carriere, A., Moreau, J. & Roux, P.P. (2010) mTORC1-activated S6K1 phosphorylates Rictor on threonine 1135 and regulates mTORC2 signaling. *Molecular and Cellular Biology*, **30**, 908–921.

Kaplan, B., Qazi, Y. & Wellen, J.R. (2014) Strategies for the management of adverse events associated with mTOR inhibitors. *Transplant Reviews (Orlando)*, **28**, 126–133.

Lee, K., Gudapati, P., Dragovic, S., Spencer, C., Joyce, S., Killeen, N., Magnuson, M.A. & Boothby, M. (2010) Mammalian target of rapamycin protein complex 2 regulates differentiation of Th1 and Th2 cell subsets via distinct signaling pathways. *Immunity*, **32**, 743–753.

Li, Y., Li, X.C., Zheng, X.X., Wells, A.D., Turka, L.A. & Strom, T.B. (1999) Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance. *Nature Medicine*, **5**, 1298–1302.

Makrigiannis, A.P. & Hoskin, D.W. (1997) Inhibition of CTL induction by rapamycin: IL-2 rescues granzyme B and perforin expression but only partially restores cytotoxic activity. *The Journal Immunology*, **159**, 4700–4707.

Manning, B.D. & Cantley, L.C. (2007) AKT/PKB signaling: navigating downstream. *Cell*, **129**, 1261–1274.

Mariotti, J., Foley, J., Jung, U., Borenstein, T., Kantardzic, N., Han, S., Hanson, J.T., Wong, E., Buxhoeveden, N., Trepel, J.B., Fojo, A.T., Telford, W. & Fowler, D.H. (2008) Ex vivo rapamycin generates apoptosis-resistant donor Th2 cells that persist in vivo and prevent hemopoietic stem cell graft rejection. *The Journal Immunology*, **180**, 89–105.

Marty, F.M., Bryar, J., Browne, S.K., Schwarzberg, T., Ho, V.T., Bassett, I.V., Koreth, J., Alyea, E.P., Soiffer, R.J., Cutler, C.S., Antin, J.H. & Baden, L.R. (2007) Sirolimus-based graft-versus-host disease prophylaxis protects against cytomegalovirus reactivation after allogeneic hematopoietic stem cell transplantation: a cohort analysis. *Blood*, **110**, 490–500.

Mortensen, D.S., Sapienza, J., Lee, B.G., Perrin-Ninkovic, S.M., Harris, R., Shevlin, G., Parnes, J.S., Whitefield, B., Hickman, M., Khambatta, G., Bisonette, R.R., Peng, S., Gamez, J.C., Leisten, J., Narla, R.K., Fultz, K.E. & Sankar, S. (2013) Use of core modification in the discovery of CC214-2, an orally available, selective inhibitor of mTOR kinase. *Bioorganic & Medicinal Chemistry Letters*, **23**, 1588–1591.

Nikolaeva, N., Bemelman, F.J., Yong, S.L., van Lier, R.A. & ten Berge, I.J. (2006) Rapamycin does not induce anergy but inhibits expansion and differentiation of alloreactive human T cells. *Transplantation*, **81**, 445–454.

Nosrati, N., Kapoor, N.R. & Kumar, V. (2014) Combinatorial action of transcription factors orchestrates cell cycle-dependent expression of the ribosomal protein genes and ribosome biogenesis. *The FEBS Journal*, **281**, 2339–2352.

Obar, J.J. & Lefrancois, L. (2010) Early events governing memory $CD8^+$ T-cell differentiation. *International Immunology*, **22**, 619–625.

Pascual, J. (2007) Post-transplant lymphoproliferative disorder–the potential of proliferation signal inhibitors. *Nephrology, Dialysis, Transplantation: Official Publication of the European Dialysis and Transplant Association – European Renal Association*, **22**(Suppl. 1), i27–i35.

Pearce, E.L., Mullen, A.C., Martins, G.A., Krawczyk, C.M., Hutchins, A.S., Zediak, V.P., Banica, M., DiCioccio, C.B., Gross, D.A., Mao, C.A., Shen, H., Cereb, N., Yang, S.Y., Lindsten, T., Rossant, J., Hunter, C.A. & Reiner, S.L. (2003) Control of effector $CD8^+$ T cell function by the transcription factor Eomesodermin. *Science*, **302**, 1041–1043.

Perez-Simón, J.A., Martino, R., Parody, R., Cabrero, M., Lopez-Corral, L., Valcarcel, D., Martinez, C., Solano, C., Vazquez, L., Marquez-Malaver, F.J., Sierra, J. & Caballero, D. (2013) The combination of sirolimus plus tacrolimus improves outcome after reduced-intensity conditioning, unrelated donor hematopoietic stem cell transplantation compared with cyclosporine plus mycofenolate. *Haematologica*, **98**, 526–532.

Powell, J.D., Lerner, C.G. & Schwartz, R.H. (1999) Inhibition of cell cycle progression by rapamycin induces T cell clonal anergy even in the presence of costimulation. *The Journal Immunology*, **162**, 2775–2784.

Rao, R.R., Li, Q., Odunsi, K. & Shrikant, P.A. (2010) The mTOR kinase determines effector versus memory $CD8^+$ T cell fate by regulating the expression of transcription factors T-bet and Eomesodermin. *Immunity*, **32**, 67–78.

Rodrik-Outmezguine, V.S., Chandarlapaty, S., Pagano, N.C., Poulikakos, P.I., Scaltriti, M., Moskatel, E., Baselga, J., Guichard, S. & Rosen, N. (2011) mTOR kinase inhibition causes feedback-dependent biphasic regulation of AKT signaling. *Cancer Discovery*, **1**, 248–259.

Sarbassov, D.D., Guertin, D.A., Ali, S.M. & Sabatini, D.M. (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science*, **307**, 1098–1101.

Sarbassov, D.D., Ali, S.M., Sengupta, S., Sheen, J.H., Hsu, P.P., Bagley, A.F., Markhard, A.L. & Sabatini, D.M. (2006) Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Molecular Cell*, **22**, 159–168.

Slavik, J.M., Lim, D.G., Burakoff, S.J. & Hafler, D.A. (2004) Rapamycin-resistant proliferation of $CD8^+$ T cells correlates with p27kip1 down-regulation and bcl-xL induction, and is prevented by an inhibitor of phosphoinositide 3-kinase activity. *The Journal of Biological Chemistry*, **279**, 910–919.

Sparks, C.A. & Guertin, D.A. (2010) Targeting mTOR: prospects for mTOR complex 2 inhibitors in cancer therapy. *Oncogene*, **29**, 3733–3744.

Sun, S.Y., Rosenberg, L.M., Wang, X., Zhou, Z., Yue, P., Fu, H. & Khuri, F.R. (2005) Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. *Cancer Research*, **65**, 7052–7058.

Thoreen, C.C., Kang, S.A., Chang, J.W., Liu, Q., Zhang, J., Gao, Y., Reichling, L.J., Sim, T., Sabatini, D.M. & Gray, N.S. (2009) An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. *The Journal of Biological Chemistry*, **284**, 8023–8032.

Tomasoni, R., Basso, V., Pilipow, K., Sitia, G., Saccani, S., Agresti, A., Mietton, F., Natoli, G., Colombetti, S. & Mondino, A. (2011) Rapamycin-sensitive signals control TCR/CD28-driven Ifng, Il4 and Foxp3 transcription and promoter region methylation. *European Journal of Immunology*, **41**, 2086–2096.

Tomiyama, H., Takata, H., Matsuda, T. & Takiguchi, M. (2004) Phenotypic classification of human $CD8^+$ T cells reflecting their function: inverse correlation between quantitative expression of CD27 and cytotoxic effector function. *European Journal of Immunology*, **34**, 999–1010.

Turner, A.P., Shaffer, V.O., Araki, K., Martens, C., Turner, P.L., Gangappa, S., Ford, M.L., Ahmed, R., Kirk, A.D. & Larsen, C.P. (2011) Sirolimus enhances the magnitude and quality of viral-specific $CD8^+$ T-cell responses to vaccinia virus vaccination in rhesus macaques. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons*, **11**, 613–618.

Waickman, A.T. & Powell, J.D. (2012) Mammalian target of rapamycin integrates diverse inputs to guide the outcome of antigen recognition in T cells. *The Journal of Immunology*, **188**, 4721–4729.

Wan, X., Harkavy, B., Shen, N., Grohar, P. & Helman, L.J. (2007) Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism. *Oncogene*, **26**, 1932–1940.

Wang, Y., Yu, Q., He, X., Romigh, T., Altemus, J. & Eng, C. (2014) Activation of AR sensitizes breast carcinomas to NVP-BEZ235's therapeutic effect mediated by PTEN and KLLN upregulation. *Molecular Cancer Therapeutics*, **13**, 517–527.

Wullschleger, S., Loewith, R. & Hall, M.N. (2006) TOR signaling in growth and metabolism. *Cell*, **124**, 471–484.

Zhou, H.Y. & Huang, S.L. (2012) Current development of the second generation of mTOR inhibitors as anticancer agents. *Chinese Journal of Cancer*, **31**, 8–18.