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Differential effects of inhibitors of the PI3K/mTOR pathway on the expansion and functionality of regulatory T cells

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ABSTRACT

The PI3K/mTOR pathway is commonly deregulated in cancer. mTOR inhibitors are registered for the treatment of several solid tumors and novel inhibitors are explored clinically. Notably, this pathway also plays an important role in immunoregulation. While mTOR inhibitors block cell cycle progression of conventional T cells (Tconv), they also result in the expansion of $CD4^+CD25^{hi}FOXP3^+$ regulatory T cells (Tregs), and this likely limits their clinical antitumor efficacy. Here, we compared the effects of dual mTOR/PI3K inhibition (using BEZ235) to single PI3K (using BKM120) or mTOR inhibition (using rapamycin and everolimus) on Treg expansion and functionality. Whereas rapamycin, everolimus and BEZ235 effected a relative expansion benefit for Tregs and increased their overall suppressive activity, BKM120 allowed for similar expansion rates of Tregs and Tconv without altering their overall suppressive activity. Therefore, PI3K inhibition alone might offer antitumor efficacy without the detrimental selective expansion of Tregs associated with mTOR inhibition.

1. Introduction

$CD4^+CD25^{hi}FoxP3^+$ regulatory T cells (Tregs) represent a functionally distinct lineage of immunoregulatory T cells that have been shown to be important regulators of immunological tolerance [1]. Tregs are critically dependent on the transcription factor FoxP3 [2], and play a diverse but crucial role in autoimmunity, transplantation and cancer [3]. They can be categorized in three subsets, thymus derived Tregs (tTregs) which were previously named natural Tregs, peripherally derived Tregs (pTregs) and *in vitro*-induced Tregs (iTregs) together previously named induced Tregs [4]. Besides CD3, CD4, CD25 and FoxP3, the absence of the surface marker CD127 and the presence of several other markers like CTLA-4 and Helios can be used to define Tregs [5].

Previous reports have shown that for the *in vitro* culture of Tregs it is preferable to add rapamycin, since this would lead to a preferential expansion of Tregs over conventional T cells (Tconv) [6–8], possibly due to selective apoptosis of conventional T cells [9]. In addition, Treg enriched cell populations cultured in the presence of rapamycin retained stable FoxP3 expression after *in vivo* transfer in a murine model [10]. Moreover, *in vivo* treatment with rapamycin was shown to lead to the expansion of Tregs [11].

Since the mTOR pathway is commonly deregulated in cancer and mTOR inhibitors have shown efficacy in the treatment of metastatic renal cell cancer [12], pancreatic neuroendocrine tumor (pNET) [13] and advanced hormone receptor-positive HER2-negative breast cancer [14], mTOR inhibitors are now widely used in the treatment of cancer. However, this could result in the detrimental expansion of Tregs thereby potentially limiting their clinical antitumor effect.

At this moment, novel inhibitors of the PI3K/mTOR pathway (like BEZ235 and BKM120) are being explored clinically [15], though their immunological effects have not been extensively studied. Therefore we set out to study the effects of dual PI3K/mTOR inhibition compared to single inhibition of PI3K or mTOR on the expansion and functionality of Treg enriched cell populations. Our data indicate that whereas single mTOR inhibition and dual PI3K/mTOR inhibition both effected a relative expansion benefit for $CD25^{hi}FoxP3^+$ Tregs and increased overall immunosuppressive activity, the PI3K inhibitor BKM120 allowed for similar expansion rates of Tregs and Tconv and did not alter the overall immunosuppressive activity. In keeping with this observation, BKM120 also induced lower, although not significant, levels of the tTreg-related markers CTLA-4 and Helios than the mTOR single or PI3K/mTOR dual inhibitors. Therefore, inhibition of PI3K might offer antitumor efficacy without the detrimental selective Treg expansion that is associated with the downstream inhibition of mTOR. Apart from potentially increasing the antitumor efficacy of inhibitors of the PI3K/mTOR

Abbreviations: iTregs, *in vitro*-induced Tregs; LAP, Latency Associated Peptide; PD-1, programmed death-1; pNET, pancreatic neuroendocrine tumor; PTEN, phosphatase and tensin homolog deleted on chromosome 10; pTregs, peripherally derived Tregs; Tconv, conventional T cells; Tregs, $CD4^+CD25^{hi}FOXP3^+$ regulatory T cells; tTregs, thymus derived Tregs.

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pathway, this knowledge could be relevant when considering combination therapy of inhibitors of this pathway with immunotherapeutic approaches.

2. Materials and methods

2.1. Cell purification

Peripheral Blood Mononuclear Cells (PBMC) were isolated from buffy coats or heparinized blood of healthy donors by density-gradient centrifugation with Lymphoprep (Axis-Shield, Olso, Norway). $CD4^+$ T cells were isolated using the untouched $CD4^+$ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's protocol with a mean (±SEM) purity of 93.9% (±2.3). Next, $CD4^+CD25^+$ cells were isolated over two consecutive magnetic columns using CD25 MicroBeads (Miltenyi Biotec) according to the manufacturer's protocol with a mean (±SEM) purity of 87% (±2.1) $CD4^+CD25^+$ cells and 63.8% (±2.9) $CD25^+FoxP3^+$ cells.

2.2. Cell cultures

MACS purified $CD4^+CD25^+$ T cells were cultured according to a modified version of a previously described protocol by Battaglia et al. [16] $CD4^+CD25^+$ T cells were plated at 0.1×10^6/ml in RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 100 I.E./ml sodium penicillin (Astellas Pharma, Leiden, the Netherlands), 100 μg/ml streptomycin sulphate (Radiumfarma-Fisiofarma, Napels, Italy), 2.0 nM L-glutamine (Life Technologies, Bleiswijk, the Netherlands), 10% pooled human AB serum (MP Biomedicals, Ohio, USA) and 0.02 mM pyruvic acid (Sigma, St. Louis, USA), hereafter referred to as culture medium, with anti-CD3/anti-CD28 Ab-coated magnetic Dynabeads (CD3/CD28 beads, Dynal Biotech/Invitrogen Life Technologies) with a bead to cell ratio of 1:2 in the presence or absence of rapamycin (Calbiochem, Merck Millipore, Darmstadt, Germany), the mTOR inhibitor RAD001 (everolimus), the dual PI3K/mTOR inhibitor BEZ235 or the single pan PI3K inhibitor BKM120 (received from Novartis, Basel, Switzerland) at the indicated concentrations. At day 2 post activation IL-2 (500 IU/mL, Proleukin, Novartis, Arnhem, The Netherlands) was added. Cultures were split and provided with fresh medium containing IL-2 with or without drugs on day 6 and day 9. On day 10 cells were harvested, washed and rested in culture medium containing 5% pooled human AB serum and low dose IL-2 (50 IU/mL). After three days cells were harvested and used for functional and phenotypic analysis. In the cultures, Tregs were defined as $CD3^+CD4^+CD25^{hi}FoxP3^+$ cells and Tconv were defined as all other $CD3^+CD4^+$ cells present in the cultures.

2.3. Flow cytometry

Cells were analyzed by flow cytometry using FITC labeled antibodies against CD4 and CD25 (both BD Biosciences, New Jersey, USA), PE labeled antibodies against CD147 (eBioscience, San Diego, USA), CD45RA (BD Biosciences) CD121b and Tim-3 (both R&D systems, Minneapolis, USA), PerCP-Cy5.5 labeled antibodies against CD3 and CD4 (BD Biosciences) and APC labeled antibodies against CD25 (BD Biosciences), CCR7, LAP (both R&D systems), and PD-1 (BD Biosciences). Stainings were performed in PBS supplemented with 0.1% BSA and 0.02% sodium-azide for 30 min at 4 °C.

Intracellular stainings were performed after fixation and permeabilization using a fixation/permeabilization kit according to manufacturer's protocol (eBioscience). FoxP3 was stained with either PCH101 PE (eBioscience) or 259D Alexa Fluor 488 (Biolegend, San Diego, USA) anti-FoxP3 mAbs. Intracellular stainings for Helios, CTLA-4 and Ki-67 were performed using FITC labeled Helios (Biolegend) and PE-labeled CTLA-4 (BD Biosciences) and Ki-67 (BD Biosciences). Before intracellular IL-2 staining, cells were stimulated for 4 h with 50 ng/mL PMA and 500 ng/mL ionomycin in the presence of brefeldin A (1:500; Golgiplug, BD Biosciences) and subsequently stained for CD25, CD4, FoxP3 and IL-2 (BD Biosciences), using the eBioscience fixation/permeabilization kit. Live cells were gated based on forward and side scatter and analyzed on a BD FACSCalibur (BD Biosciences) using CellQuest or Kaluza Analysis Software (Beckman Coulter).

2.4. Western blot

The expression of PI3K/mTOR pathway proteins was determined in freshly isolated $CD4^+CD25^+$ T cells, cultured in the presence of rapamycin, RAD001, BEZ235 or BKM120, IL-2100 IU/mL and anti-CD3/anti-CD28 Ab-coated magnetic Dynabeads. After 24 h cells were harvested, washed with PBS and lysed with Sample Diluent Concentrate 2 (R&D systems) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Life Technologies). After sonication (Soniprep 150, amplitude 18, for 3 times 5 s with 10 s time intervals, on ice) cells were centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant was collected and protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Life Technologies). Equal amounts (10–20 μg) of protein from each sample were separated on 10% SDS polyacrylamide gels and subsequently transferred to PVDF membranes (Immobilon-FL, Millipore, Carrigtohill, Ireland). Membranes were incubated with primary antibodies phospho-Akt (Ser473, mouse) total Akt (rabbit), phospho-p70S6K (Thr389, mouse), and total p-70S6K (rabbit, all from Cell Signaling, Danvers, Massachusetts) as specified in the manufacturer's protocol, and specific binding was visualized by using species-specific HRP conjugated immunoglobulin G, followed by enhanced chemiluminescent detection and exposure to ECL X-ray film. Anti-β-actin (A5441) (Sigma-Aldrich, St. Louis, USA) was used as a loading control. Bands were quantified by analysis of films using the Image J Program, and values were corrected using the corresponding β-actin protein band and normalized to untreated samples.

2.5. Suppression assay

The capacity of Treg enriched cell populations to suppress proliferation of allogeneic $CD8^+$ T responder cells was determined by labeling responder T cells with 1 μM CFSE (Sigma-Aldrich) for a subsequent culture in a 96 well round-bottom plate at a concentration of 5×10^4 cells/well in culture medium in the presence of 1 μg/mL anti-CD3 mAb, 1 μg/mL anti-CD28 mAb (clones 16A9 and 15E8, kindly provided by Dr. René van Lier, Sanquin, Amsterdam, the Netherlands) and 20 U/mL rhIL-2 with or without the addition of Treg enriched cell populations in a Treg enriched cell/T responder ratio of 1:1, 1:2 and 1:4. After 4 days of co-culture, cells were stained with APC labeled CD8 (BD Biosciences) and proliferation of $CD8^+$ responder T cells was analyzed by assessing CFSE dilution. Relative proliferation was calculated by the equation % proliferation = (% responder T cells that proliferated when cultured in the presence of Treg enriched cells/% responder T cells that proliferated when cultured alone) × 100 and relative suppression was calculated by the equation % suppression = 100 – [(% responder T cells that proliferated when cultured in the presence of Treg enriched cells/% responder T cells that proliferated when cultured alone) × 100].

2.6. Statistical analysis

One-way or two-way repeated measures ANOVA was used to determine statistical significance of differences between groups with Bonferroni or Dunn's post-tests. Findings were considered statistically significant when *p*-values were ≤0.05, as indicated with asterisks (* $p \le 0.05$, ** $p < 0.01$, *** $p < 0.001$). Statistical analyses were performed using GraphPad Prism software (version 5.02, 2008).

3. Results

3.1. Blocking efficacy of PI3K/mTOR inhibitors

$CD4^+CD25^+$ Treg enriched cell populations with a mean CD4 purity (±SEM) of 93.9% (±2.3), and a mean FoxP3 purity (±SEM) of 63.8% (±2.9) were cultured in the presence of three PI3K/mTOR pathway inhibitors that are either clinically approved or in clinical testing. The mTOR inhibitor RAD001 (everolimus), the dual PI3K/mTOR inhibitor BEZ235 and the single pan PI3K inhibitor BKM120 were used in order to investigate the effect of inhibiting different proteins of the PI3K/mTOR pathway on the *in vitro* expansion and functionality of Treg enriched cell populations. Since these inhibitors were not previously described as used for the culture of Tregs or other T cells, Treg enriched cell populations were first cultured in a concentration range of each of these drugs to assess what concentration resulted in maximal purity and highest intensity of FoxP3 expression. For RAD001, BEZ235 and BKM120 this optimal concentration was 100 nM, 1 μM and 1 μM respectively (data not shown). The effects of these three inhibitors were then compared with rapamycin (100 nM), the mTOR inhibitor most commonly used for the *in vitro* culture and expansion of Tregs [6–9], and to a condition where Treg enriched cell populations were cultured in the presence of medium alone (control condition). To confirm the blocking efficacy of the inhibitors at the selected concentrations, western blots were performed. The blocking efficacy on PI3K was assessed by the amount of phosphorylation of the downstream protein Akt while the blocking efficacy of the mTOR protein was measured by assessing phosphorylation of one of its downstream proteins p70S6K. As shown in Fig. 1, both the dual PI3K/mTOR inhibitor BEZ235 and the single pan PI3K inhibitor BKM120 efficiently blocked PI3K as shown by reduced expression of phosphorylated Akt compared to the control condition (Fig. 1A upper panel and Fig. 1B). All inhibitors effected efficient blocking of mTOR as shown by reduced expression of the phosphorylated form of the downstream protein p70S6K compared to the control condition (Fig. 1A fourth panel and Fig. 1C). In conclusion, the inhibitors were shown to effectively block their targets in the tested concentrations and therefore these concentrations were used for subsequent experiments.

3.2. mTOR pathway inhibition results in expansion of $CD25^{hi}FoxP3^+$ Tregs

To investigate the effect of rapamycin, RAD001, BEZ235 and BKM120 on the expansion of Treg enriched cell populations, $CD4^+CD25^+$ T cells were cultured for two weeks in the presence of these inhibitors and anti-CD3/anti-CD28 Ab-coated magnetic Dynabeads (CD3/CD28 beads, Dynal Biotech/Invitrogen Life Technologies) and IL-2 as described in the materials and methods section. After two weeks the percentage of $CD25^{hi}FoxP3^+$ Tregs was determined for each drug condition. As illustrated in Fig. 2A, compared to the control condition in which Treg enriched cell populations were cultured without the addition of a specific inhibitor, the presence of any PI3K/mTOR pathway inhibitor resulted in a higher purity of $CD25^{hi}FoxP3^+$ T cells, with a mean (±SEM) percentage of 42.1% (±3.7) in the control condition, 72.3% (±2.4) with rapamycin ($p < 0.001$), 76.5% (±2.8) with RAD001 ($p < 0.001$), 71.3% (±4.2) with BEZ235 ($p < 0.001$), and 61.1% (±3.6) with BKM120 ($p < 0.001$) (Fig. 2B). The MFI of Foxp3 (Fig. 2C) followed the same trend as shown for the percentages. Interestingly, the single pan PI3K inhibitor BKM120 resulted in a lower purity of $CD25^{hi}FoxP3^+$ cells compared to culture conditions where either the mTOR protein alone was inhibited (*i.e.* in rapamycin and RAD001 conditions), or where the dual PI3K/mTOR inhibitor BEZ235 was used, indicating a more dominant role for mTOR in the selective expansion of Tregs.

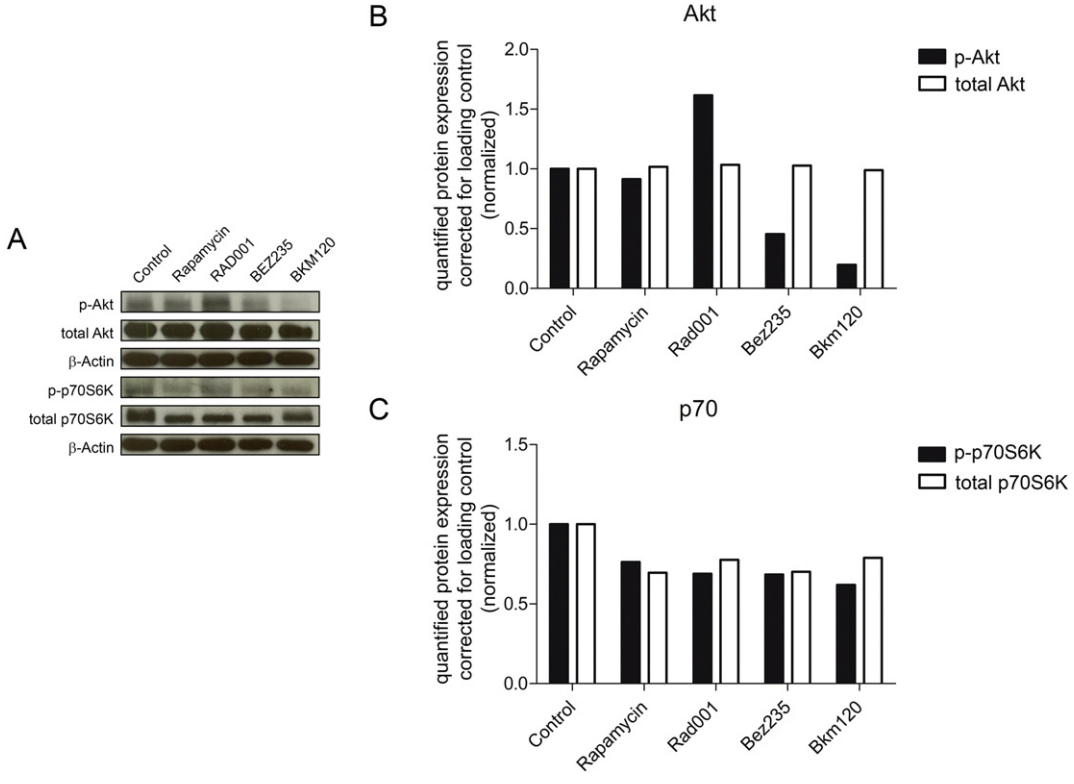

Fig. 1. Western blots of mTOR signaling proteins. The blocking efficacy of the four PI3K/mTOR pathway inhibitors were determined in freshly isolated $CD4^+CD25^+$ T cells cultured for 24 h in the presence of medium (control), rapamycin 100 nM, RAD001 100 nM, BEZ235 1 μM or BKM120 1 μM, IL-2100 IU/mL and anti-CD3/anti-CD28 Ab-coated magnetic Dynabeads. A, Immunoblots for phospho-Akt (Ser473), total Akt, phospho-p70S6K (Thr389) and total p70S6K. β-actin was used as a loading control. B–C, Bands were quantified by analysis of films using the Image J Program, and values were corrected using the corresponding β-actin protein band and normalized to untreated samples; B, quantification of phospho-Akt and total Akt; C, quantification of phospho-p70S6K and total p70S6K. Data shown are representative for 2 independent experiments.

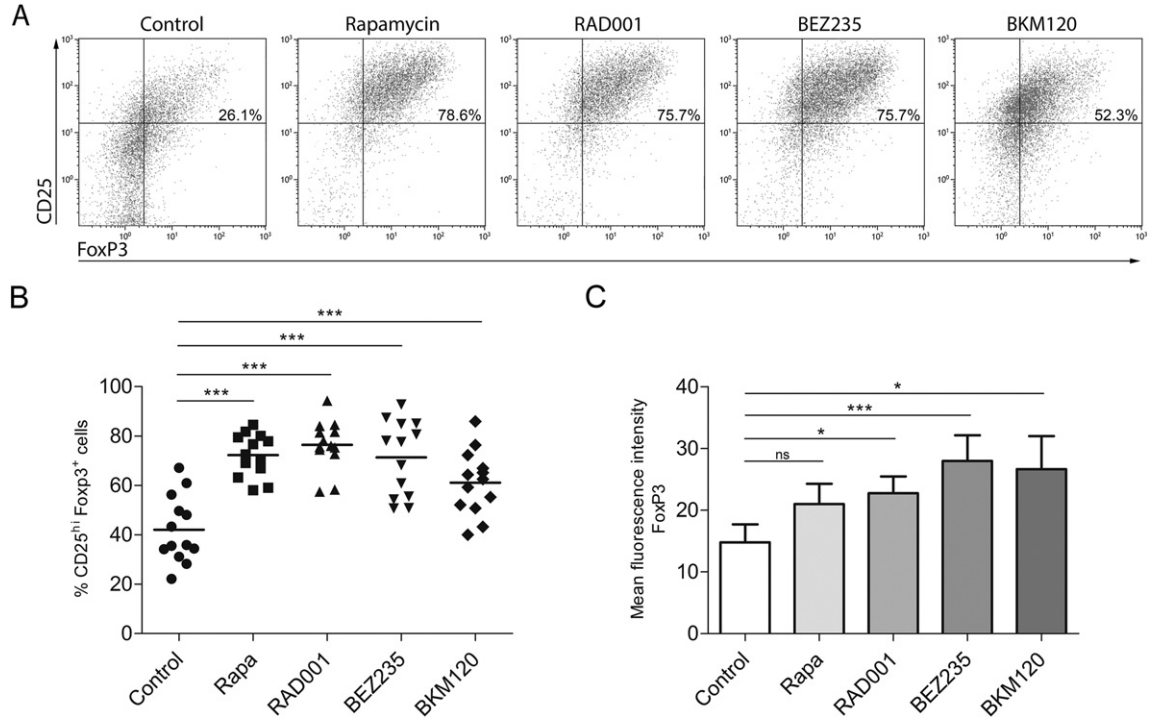

Fig. 2. mTOR inhibition results in expansion of Tregs. A, Representative dot plots illustrating the percentages of $CD25^{hi}FoxP3^{+}$ Tregs determined after two weeks of culture of Treg enriched cell populations with IL-2 and anti-CD3/anti-CD28 Ab-coated magnetic Dynabeads in the presence of medium (control), rapamycin, RAD001, BEZ235 or BKM120. B–C, The percentages (B) of $CD25^{hi}FoxP3^{+}$ Tregs were assessed by flow cytometry and the mean fluorescence intensity (C) of FoxP3 was determined. Means + SEM are shown; *p*-values are indicated with asterisks; * $p \leq 0.05$, *** $p < 0.001$; $n = 13$; One way repeated measures ANOVA with Bonferroni post-test.

3.3. mTOR inhibition results in a relative expansion benefit for Tregs

The *in vitro* culture of Tregs from peripheral blood is complicated by the fact that both CD4 and CD25, surface markers commonly used for isolation of $CD4^{+}CD25^{+}$ Tregs, can also be expressed by activated Tconv resulting in a starting population containing both cell types. However, previous studies have shown that addition of rapamycin to $CD4^{+}CD25^{+}$ T cell cultures leads to a selective expansion of Tregs, due to the apoptosis of Tconv in the presence of rapamycin [9]. To assess whether the various PI3K/mTOR pathway inhibitors resulted in a preferential expansion of Tregs over Tconv cells, the expansion factors of absolute numbers of both Tregs, defined as $CD25^{hi}FoxP3^{+}$ T cells, and Tconv, defined as all other cells present in the cultures, were calculated and a ratio was determined. As shown in Fig. 3, while culturing Treg enriched cell populations in the presence of medium and IL-2 alone (control condition) resulted in the predominant expansion of Tconv (mean ratio of 0.5 (±0.07)), both single mTOR inhibition using rapamycin (mean ratio of 1.6 (±0.2), $p < 0.001$) or RAD001 (mean ratio of 2.2 (±0.3), $p < 0.001$) and dual PI3K/mTOR inhibition using BEZ235 (mean ratio of 2.3 (±0.6), $p < 0.01$) supported the preferential expansion of Tregs. Interestingly, culturing Treg enriched cell populations in the presence of BKM120 resulted in similar expansion rates of Tregs and Tconv (mean ratio of 1.1 (±0.2), $p =$ n.s.), indicating that inhibition of the mTOR pathway upstream of the protein kinase mTOR diminishes the selective expansion benefit for Tregs.

3.4. Phenotypic analysis of expanded Tregs

To investigate the phenotypic characteristics of Tregs cultured in the presence of the different PI3K/mTOR pathway inhibitors, $CD25^{hi}FoxP3^{+}$ Tregs were analyzed for several Treg-associated markers, as shown in Fig. 4. The tTreg markers CTLA-4 and Helios were more abundantly (though not statistically significant) expressed by Tregs cultured in the presence of an mTOR pathway inhibitor compared to Tregs cultured in the control condition, with similar expression levels of KI-67. An analysis of the expression of two markers associated with activated Tregs, *i.e.* CD147 and CD121b [17,18], revealed that both markers were equally expressed by the $CD25^{hi}FoxP3^{+}$ Tregs in all five culture conditions. Interestingly, while we indeed observed high expression of CD147, the level of CD121b was substantially lower than was expected based on a previous report studying activated human regulatory T cells [17,18]. In addition, the expression of Latency Associated Peptide (LAP), a marker frequently expressed by activated Tregs and associated with cell contact-dependent immunosuppression was determined [18–20].

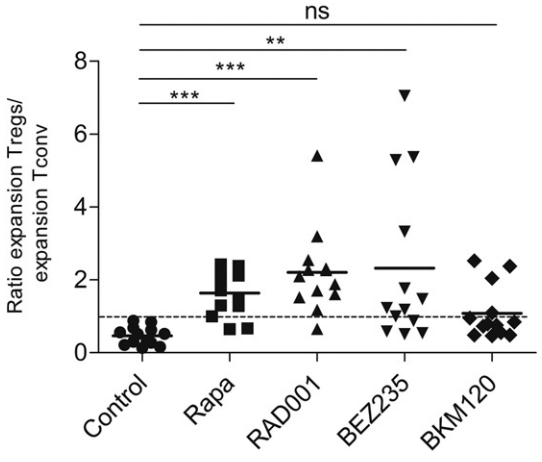

Fig. 3. mTOR inhibition results in a relative expansion benefit for Tregs. Treg enriched cell populations were cultured for two weeks with IL-2 and anti-CD3/anti-CD28 Ab-coated magnetic Dynabeads in the presence of medium (control), rapamycin, RAD001, BEZ235 or BKM120. Expansion factors of absolute numbers of both Tregs, defined as $CD25^{hi}FoxP3^{+}$ T cells, and Tconv, defined as all other cells present in the cultures, were calculated and a ratio was determined. Means + SEM are shown; *p*-values are indicated with asterisks; ** $p < 0.01$, *** $p < 0.001$; $n = 13$; One way repeated measures ANOVA with Friedman post-test.

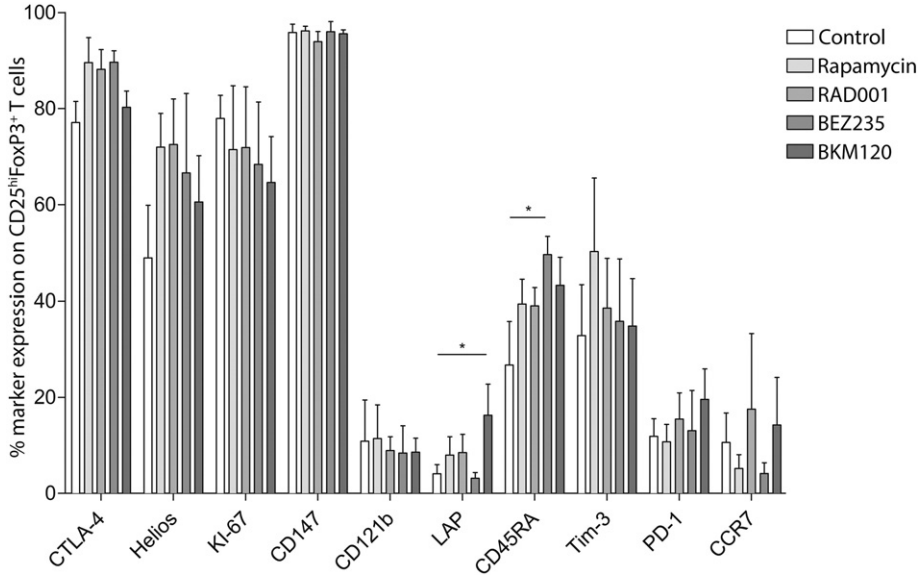

Fig. 4. Phenotypic analysis of expanded Tregs. Treg enriched cell populations were cultured for two weeks with IL-2 and anti-CD3/anti-CD28 Ab-coated magnetic Dynabeads in the presence of medium (control), rapamycin, RAD001, BEZ235 or BKM120. After two weeks the expression of several Treg associated markers was assessed on $CD25^{hi}FoxP3^{+}$ Tregs by flow cytometry. Means + SEM are shown; *p*-values are indicated with asterisks; * $p \leq 0.05$; $n = 3$–4; One-way repeated measures ANOVA with Bonferroni post-tests.

Analyses revealed a more abundant, but still relatively low expression of LAP on $CD25^{hi}FoxP3^{+}$ Tregs cultured in the presence of BKM120. Compared to the control condition, CD45RA was significantly higher expressed by Tregs cultured in the presence of BEZ235 indicating the presence of more phenotypically naïve $CD25^{hi}FoxP3^{+}$ Tregs [21]. No major differences were observed between the different mTOR pathway inhibitors with respect to the expression of the inhibitory receptors Tim-3 and programmed death-1 (PD-1) or the lymphoid migratory chemokine receptor CCR7.

3.5. Cytokine production by expanded Tregs

Tregs are known to be highly dependent on IL-2 [22] and can, in contrast to Tconv, hardly produce this cytokine themselves [23]. To evaluate the production of IL-2 in our cultures, intracellular stainings for IL-2 were performed. To discriminate between activated Tconv and Tregs, two populations were gated based on the gating strategy that is shown in Fig. 2A (*i.e.* activated Tconv: $CD25^{hi}FoxP3^{-}$ T cells (upper left quadrants); Treg: $CD25^{hi}FoxP3^{+}$ T cells (upper right quadrants)). Representative dot plots of IL-2 production in $CD25^{hi}FoxP3^{-}$ Tconv and $CD25^{hi}FoxP3^{+}$ Tregs cultured in the presence of medium (control) or rapamycin are shown in Fig. 5A. As shown in Fig. 5B, in all five conditions intracellular IL-2 was primarily produced by Tconv, with only very low levels being detectable in Tregs. No significant differences between the different mTOR pathway inhibitors were observed with respect to intracellular IL-2 production.

3.6. mTOR inhibition results in Tregs with suppressive functionality

To evaluate the capacity of the Treg enriched cell populations, cultured in the presence of one of the four different mTOR pathway inhibitors, to suppress other T cells, Treg enriched cell populations were cultured for two weeks with anti-CD3/anti-CD28 Ab-coated magnetic Dynabeads and IL-2. Subsequently Treg enriched cell populations were harvested and co-cultured with CFSE labeled $CD8^{+}$ responder T cells (Fig. 6A). Cell division of the responder T cells was assessed after a 4-day co-culture period of responder T cells with different ratios of Treg enriched cell populations, in the presence of 1 μg/mL anti-CD3 mAb, 1 μg/mL anti-CD28 mAb and 20 U/mL IL-2 (Fig. 6B). Cells cultured in the presence of Dynabeads and IL-2 alone (control condition, Fig. 6A lower left panel) already reduced the proliferation of responder T cells

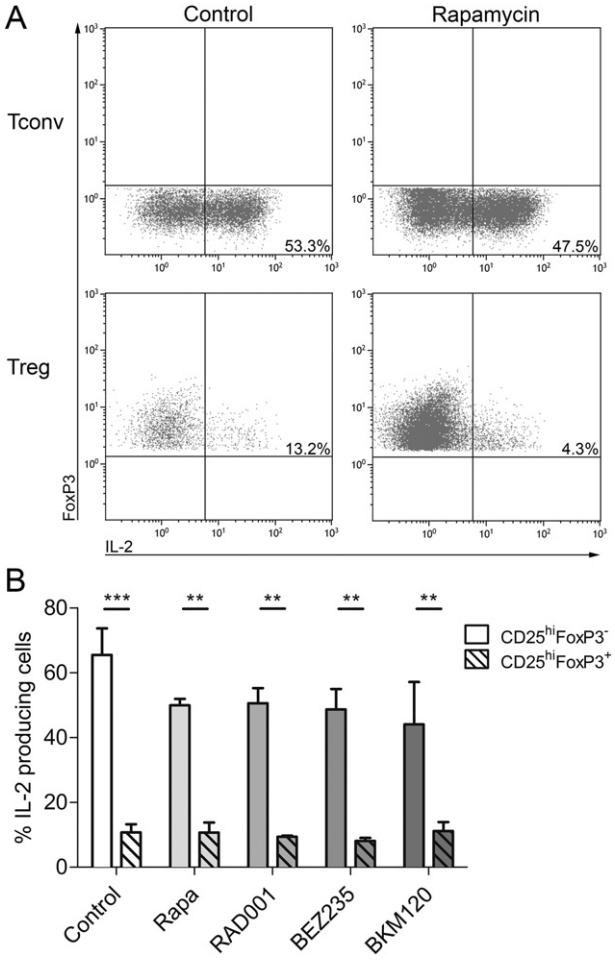

Fig. 5. IL-2 production of expanded Tregs. A, Representative dot plots of IL-2 production in $CD25^{hi}FoxP3^{-}$ Tconv and $CD25^{hi}FoxP3^{+}$ Tregs, cultured in the presence of medium (control) or rapamycin. B, Bar graph showing the percentage of IL-2 positive cells in Treg enriched cell populations cultured in medium (control), rapamycin, RAD001, BEZ235 or BKM120. Means + SEM are shown; *p*-values are indicated with asterisk; ** $p < 0.01$, *** $p < 0.001$; $n = 3$; two-way repeated measures ANOVA with Bonferroni post-test.

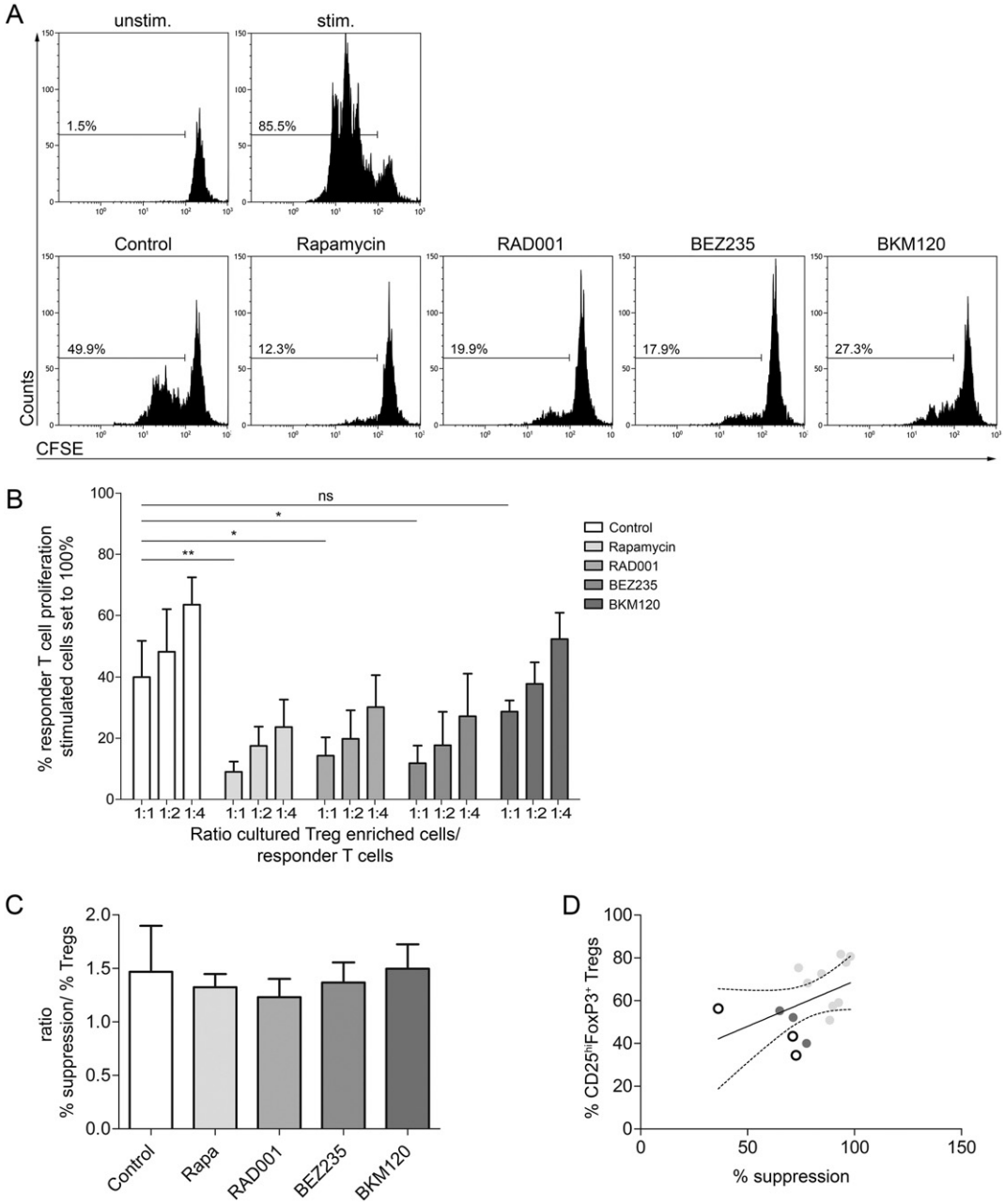

Fig. 6. mTOR inhibition results in Tregs with suppressive function and suppression is related to percentage of Tregs in expanded cultures. Treg enriched cell populations were cultured for two weeks with IL-2 and anti-CD3/anti-CD28 Ab-coated magnetic Dynabeads in the presence of medium (control), rapamycin, RAD001, BEZ235 or BKM120. Their capacity to suppress T cell proliferation was tested by measuring CFSE dilution of stimulated $CD8^+$ responder T cells using anti-CD3 mAb, anti-CD28 mAb, and IL-2. A, Representative histograms showing CFSE dilution of responder T cells in the various conditions. Unstimulated $CD8^+$ responder T cells without the addition of anti-CD3 mAb, anti-CD28 mAb, and IL-2 and $CD8^+$ responder T cells stimulated with anti-CD3 mAb, anti-CD28 mAb, and IL-2, both without the addition of cultured Treg enriched cell populations (upper two panels) were used as control conditions. Lower panels show the CFSE dilution of responder T cells cultured with Treg enriched cell populations that were generated in the presence of medium (control), rapamycin, RAD001, BEZ235 or BKM120. B, Bar graph showing the proliferation of responder T cells cultured with Treg enriched cell populations generated in the presence of medium (control), rapamycin, RAD001, BEZ235 or BKM120, relative to responder T cells alone in different ratios of cultured Treg enriched cell populations *versus* responder T cells (1:1, 1:2, 1:4). Means + SEM are shown. Statistics were performed on the 1:1 ratios of cultured Treg enriched cell populations *versus* responder T cells. *p*-values are indicated with asterisks; * $p \leq 0.05$, ** $p < 0.01$; $n = 3$; One way repeated measures ANOVA with Bonferroni post-test. C-D, Percentages of $CD25^{hi}FoxP3^+$ Tregs were assessed by flow cytometry. CFSE dilution of $CD8^+$ responder T cells was determined and represented as suppression of responder T cells relative to $CD8^+$ responder T cells stimulated with anti-CD3 mAb, anti-CD28 mAb, and IL-2. C, The ratio of the percentage of suppression and the percentage of Tregs. D, Correlation between the frequency of $CD25^{hi}FoxP3^+$ Tregs and the relative suppression of responder T cells. Each point represents an individual data point, symbols represent the different conditions *e.g.* open bullet points: no addition of an inhibitor (control ○), black bullet points: Treg enriched cell populations generated in the presence of BKM120 (●), grey bullet points: Treg enriched cell populations generated in the presence of mTOR inhibition, both single mTOR and dual PI3K/mTOR inhibitors (●). *p*-value < 0.01, $R = 0.65$.

compared to the stimulation control condition (Fig. 6A, upper right panel) in which responder T cells were stimulated in the presence of anti-CD3, anti-CD28 mAb and 20 U/mL IL-2 without the addition of other cells. This could be explained by the presence of a minor percentage of Tregs as noted in Fig. 2, combined with a competition of Tconv in the control bulk cultures with the responder T cells for growth factors and nutrients.

Treg enriched cell populations cultured in the presence of an mTOR inhibitor, either a single mTOR inhibitor (rapamycin or RAD001) or the dual PI3K/mTOR inhibitor (BEZ235), resulted in a significant decrease in

responder T cell proliferation compared to the control condition (p-value compared to control for rapamycin condition $p < 0.01$, for RAD001 and BEZ235 $p \leq 0.05$). In contrast, Treg enriched cell populations cultured in the presence of the PI3K inhibitor BKM120 were not able to suppress the proliferation of responder T cells to a similar extent as cells cultured in the presence of an mTOR inhibitor, as shown in Fig. 6B (p-value of control condition compared to BKM120 was not significant).

Interestingly, when relating the observed suppression of responder T cell proliferation to the percentage of Tregs present in the culture, we found that all four mTOR pathway inhibitors as well as the control condition showed a similar suppression per Treg (Fig. 6C), indicating that mTOR inhibition does not appear to enhance the suppressive capacity per Treg. In addition, a significant correlation between the percentage of $CD25^{hi}FoxP3^{+}$ cells within the differentially conditioned cultures and their suppressive activity (Fig. 6D) was found, indicating that inhibitors of the PI3K/mTOR pathway modulate immunosuppression *via* their effects on the frequency of Tregs.

4. Discussion

Novel PI3K/mTOR pathway inhibitors are currently under clinical investigation for the treatment of various malignancies [15], however, suppressive effects on the immune system could be detrimental and contribute to tumor survival instead of the intended tumor rejection. Therefore, studying the effect of targeted therapies on the immune system could be of additional value.

mTOR inhibitors were originally designed to target the immune system for prevention of transplant rejection by inhibiting the activation of T cells [24]. Although patients treated with these mTOR inhibitors showed reduced cancer risks [25], it is still quite remarkable that these inhibitors are now actually used in the treatment of cancer taking into account the already suppressed state of the immune system in this disease and the fact that mTOR inhibitors have been shown to induce *in vitro* [6–8] as well as *in vivo* [11] expansion of immunosuppressive Tregs which are associated with poor prognosis in cancer patients [26,27]. In addition, we found a more suppressed state of the immune system with an increase in circulating Treg frequencies in renal cell cancer patients treated with everolimus (Huijts CM et al., manuscript in preparation). As low dose metronomic cyclophosphamide has been reported to deplete Tregs in cancer patients [28], we are currently conducting a phase 1–2 clinical trial in patients with metastatic renal cell cancer in which standard second line treatment with everolimus is combined with low dose metronomic cyclophosphamide in order to assess whether preventing the potentially detrimental mTOR inhibitor associated increase in Tregs can result in an enhanced clinical antitumor efficacy of everolimus [29]. As several PI3K/mTOR pathway inhibitors are currently under development for the treatment of cancer [15], we analyzed three relatively novel PI3K/mTOR pathway inhibitors for their effects on Tregs *in vitro*. Our data show that $CD25^{hi}FoxP3^{+}$ Tregs have an expansion benefit over Tconv when cultured in the presence of an mTOR inhibitor while more upstream inhibition of the mTOR pathway using a PI3K inhibitor, leads to similar expansion rates for Tregs and Tconv. Tregs cultured in the presence of either an mTOR inhibitor, a PI3K inhibitor or a dual PI3K/mTOR inhibitor show an overall similar phenotype characterized by the expression of CTLA-4, Helios, KI-67 and CD147 with intermediate/low expression of CD121b, TIM-3, PD-1 and CCR7 and a low capacity to produce IL-2. The main difference in marker expression that was observed between the investigated inhibitors was the induction of LAP expression on Tregs cultured in the presence of BKM120. As LAP is a marker found on the surface of activated tTregs [18], it is hard to explain the more abundant expression of this marker on Tregs cultured in the presence of BKM120, since these Tregs did not show other characteristics of activated tTregs, such as an increase in suppression. However, BKM120 also induced lower, although not significantly so, levels of the tTreg-related markers CTLA-4 and Helios than the mTOR single or PI3K/mTOR dual inhibitors pointing towards the survival of tTregs in the presence of an mTOR inhibitor.

When analyzing the suppressive capacities of the Treg enriched cell populations, we observed that Treg enriched cell populations cultured in the presence of a single mTOR or dual PI3K/mTOR inhibitor displayed an increase in their overall suppressive capacity, while Treg enriched cell populations cultured in the presence of the PI3K inhibitor did not. These data indicate that the target site used for inhibiting the PI3K/mTOR pathway determines the immunosuppressive effect of these compounds. While inhibition of mTOR results in the preferential expansion of Tregs over Tconv, it is possible to shift this balance in favor of Tconv upon more upstream inhibition of the pathway, as shown by the use of the PI3K inhibitor BKM120. Whether this is due to the inability of Tregs to expand or the relative insensitivity of Tconv for PI3K inhibition remains to be elucidated.

Tregs and Tconv differ in their intracellular signaling pathways. Activation of Tconv results in downregulation of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which negatively regulates PI3K, facilitating the activation of the pathway *via* Akt and mTOR. However, Tregs are less dependent on the PI3K/mTOR pathway compared to Tconv due to the constitutive expression of PTEN, even after activation [30]. In addition, Tregs were shown to express the protein kinase PIM2 in a FoxP3 dependent manner [31], allowing them to progress through cell cycle by the use of the JAK/STAT5 pathway without the need for the mTOR pathway. This could be an explanation for the ability of Tregs to proliferate in the presence of an mTOR inhibitor while Tconv cannot. Interestingly however, we observed that culture of Treg enriched cell populations in the presence of a PI3K inhibitor resulted in similar expansion rates for Tregs and Tconv, so the proliferation benefit for Tregs seen in the presence of an mTOR inhibitor was lost in the presence of PI3K inhibition, indicating a more important role for the PI3K protein in the proliferation of Tregs. This is in line with previously published results, showing a critical role for PI3K in the function of Tregs, and in particular the p110δ isoform of PI3K [32]. Additionally, it was shown that the proliferation and differentiation of Tregs *in vivo* is dependent on PI3K activation and that inhibition of PI3K exerts a stronger effect on Tregs compared to Tconv or cytotoxic $CD8^{+}$ T cells [33].

How the proliferation and immunosuppressive function of Tregs is exactly affected by the inhibitors of the PI3K/mTOR pathway remains to be elucidated. For example, counterintuitively both upstream inhibition in the PI3K/mTOR pathway, by inhibiting PI3K [32], as well as overexpression of the more downstream protein Akt [34], have been shown to negatively impact Tregs, whereas inhibition of the downstream protein mTOR results in Treg expansion *in vitro* and *in vivo* [6,11], suggesting that there is probably a certain window in the range in the level of activation of the PI3K/mTOR pathway in which optimal Treg expansion and function is maintained (also reviewed in Ref. [35]). The apparently opposing roles of PI3K and mTOR in Tregs were proposed to be explained by a feedback loop in which mTOR inhibition results in PI3K-dependent Akt activation [36]. Importantly, in our study dual inhibition of both PI3K and mTOR by BEZ235 resulted in the preferential proliferation of Tregs (as was also observed with mTOR inhibition alone), indicating that inhibition of mTOR is dominant over PI3K inhibition in inducing expansion of Tregs. In line with this observation are data reporting comparable effects of rapamycin and BEZ235 on Treg differentiation [37]. Together, these data therefore indicate that the proposed feedback loop cannot completely explain the observed effects. Other possible explanations include direct interactions between PI3K and STAT5, as observed in hematologic malignances, whereby inhibition of PI3K can result in the inhibition of STAT5, thereby inhibiting progression through cell cycle [38]. Alternatively, some of the observed results could be explained by effects of the inhibitors on Tconv instead of Tregs. Indeed, studies have also indicated that mTOR inhibitors may lead to selective Treg expansion due to apoptosis of Tconv [9]. Furthermore, recent observations showed Tregs to be more sensitive to PI3K inhibition

compared to Tconv, which could give Tconv the opportunity to preferentially proliferate in the presence of PI3K inhibitors [36].

Interestingly, although mTOR inhibition resulted in an increase in the immunosuppressive capacity of the total Treg cell enriched population, this was caused by an increase in the frequency of Tregs and not by an increase in the suppressive capacity of individual Tregs, as we found that the suppression per Treg was similar in all culture conditions.

In conclusion, therapies targeting the PI3K/mTOR pathway can result in detrimental immunological effects that may limit the development of effective antitumor immune responses. Here we demonstrate that PI3K-targeted inhibition of the PI3K/mTOR pathway using PI3K inhibition, is not accompanied by the preferential expansion of Tregs that we and others have observed using mTOR inhibitors, and thereby do not contribute to a more immune suppressive state. Therefore, PI3K inhibitors could be a suitable, perhaps clinically more effective, alternative for mTOR inhibition in the treatment of cancer, without detrimental predominant Treg expansion.

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