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Rapamycin increases the yield and effector function of human $\gamma\delta$ T cells stimulated in vitro

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Abstract

Clinical strategies to exploit $V\gamma 2V\delta 2$ T cell responses for immunotherapy are confronted with short-term increases in cell levels or activity and the development of anergy that reduces the response to therapy with succeeding treatments. We are exploring strategies to increase the yield and durability of elicited $V\gamma 2V\delta 2$ T cell responses. One approach focuses on the mammalian target of rapamycin (mTOR), which is important for regulating T cell metabolism and function. In $V\gamma 2V\delta 2$ T cells, mTOR phosphorylates the S6K1 and eIF4EBP1 signaling intermediates after antigen stimulation. Rapamycin inhibited these phosphorylation events without impacting Akt or Erk activation, even though specific inhibition of Akt or Erk in turn reduced the activation of mTOR. The effects of rapamycin on the T cell receptor signaling pathway lead to increased proliferation of treated and antigen-exposed $V\gamma 2V\delta 2$ cells. Rapamycin altered the phenotype of antigen-specific $V\gamma 2V\delta 2$ cells by inducing a population shift from CD62L + CD69– to CD62L– CD69+, higher expression of CD25 or Bcl-2, lower levels of CCR5 and increased resistance to Fas-mediated cellular apoptosis. These changes were consistent with rapamycin promoting cell activation while decreasing the susceptibility to cell death that might occur by CCR5 or Fas signaling. Rapamycin treatment during antigen-stimulation of $V\gamma 2V\delta 2$ T cells may be a strategy for overcoming current obstacles in tumor immunotherapy.

Keywords

T cell; Rapamycin; Cancer; Immunotherapy; Gammadelta; Metabolism; Vdelta2

Introduction

$V\gamma 2V\delta 2$ T cells (also termed $V\gamma 9V\delta 2$ and here referred to as $V\delta 2$ T cells) are a subset of human peripheral $\gamma\delta$ T cells [1] that exhibit broad, MHC-unrestricted lytic activity against human tumor [2] or virally infected cells [3–5]. $V\delta 2$ T cells recognize, proliferate, release cytokine [6,7] and degranulate [8,9] in response to small phosphorylated compounds known as phosphoantigens [10], that include isopentenyl pyrophosphate (IPP), 4-hydroxy-3-dimethylallylpyrophosphate (HMBPP) and synthetic bromohydrin pyrophosphate (BrHPP) [5]. $V\delta 2$ T cells also respond to bisphosphonates (BP) which elevate intracellular levels of phosphoantigen by inhibiting the IPP-processing enzyme farnesyl pyrophosphate synthase (FPPS) [5]. Characteristics of $V\delta 2$ T cells including activation and expansion after drug treatment and cytotoxicity for human tumor cells, have encouraged efforts to exploit them

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for immunotherapy of cancer [4,5]. Clinical trials using phosphoantigens, bisphosphonates [11–14] or ex vivo-expanded and adoptively transferred V δ 2 T cells [15,16] demonstrated their value for tumor immunotherapy.

Even with promising results from early clinical studies, there are important obstacles to the wider application of V δ 2 T cell-based therapy. For example, a macaque study showed that responses to repeated BrHPP/IL2 injections declined progressively compared to the first treatment [17], suggesting that repeated administration of phosphoantigen might cause anergy, exhaustion or even death of the effector V δ 2 T cells. A similar pattern of declining response was reported for bisphosphonate treatment in prostate cancer patients [14]. Thus, therapies targeting V δ 2 T cells produce short-term responses in the face of chronic disease. Even with this limitation, objective clinical responses were achieved and there is strong motivation to continue developing this approach. If we can extend the duration of elevated V δ 2 T cell levels and function following treatment, repetitive dosing may not be necessary or may not show the detrimental anergizing effect. Here, we report the promising results that rapamycin may potentiate and increase the duration of V δ 2 cell responses to phosphoantigen immunotherapy.

Rapamycin (Sirolimus) was discovered in a soil sample from Easter Island as a product of the bacterium *Streptomyces hygroscopicus* [18]. This macrolide inhibits cell proliferation and has potent immunosuppressive activity when used at higher doses. It is used currently in solid organ transplantation, especially for kidney transplants [19]. Curiously, the use of rapamycin for immunosuppression is also accompanied by increased inflammatory events including lymphocytic alveolitis, interstitial pneumonitis, and severe forms of glomerulonephritis [20–23], showing that this drug has complex effects on the human immune system.

Rapamycin specifically inhibits a serine/threonine protein kinase termed mammalian target of rapamycin (mTOR) [24]. The mTOR kinase is present in two cellular protein complexes; mTOR complex1 (mTORC1) and mTORC2, which have distinct subunit compositions, substrates and mechanisms of activation [24,25]. mTORC1 is highly sensitive to inhibition by rapamycin, whereas mTOR in mTORC2 is resistant to the drug [24]. The best-characterized substrates for mTORC1 are S6 kinase 1 (S6K1) and the eukaryotic initiation factor 4E-binding protein-1 (EIF4EBP1) [24]. The variety of rapamycin effects on immunity are receiving increased attention [26], including inhibition of type I interferon production by plasmacytoid dendritic cells [27], shaping the maturation and function of myeloid dendritic cells [28], modulating T lymphocyte trafficking [29], regulating Foxp3 expression in regulatory T cells [30], enhancing memory CD8 T cell differentiation in virus infection [31] and modulating CCR5 levels [32]. The effects of rapamycin on V δ 2 T cells are yet uncharacterized.

In the present study, we found that rapamycin altered IPP/IL2-induced proliferation by increasing the yield and functionality of V δ 2 T cells. Our results suggest that rapamycin might be effective for improving $\gamma\delta$ T cell based immunotherapy and should be tested in preclinical models.

Materials and methods

PBMC separation

Whole blood was obtained from healthy human volunteers who provided written informed consent. Protocols were approved by the Institutional Review Board at the University of Maryland, Baltimore. Total lymphocytes were separated from heparinized peripheral blood by density gradient centrifugation (Ficoll-Paque; Amersham Biosciences). Peripheral-blood

mononuclear cells (PBMC) and TU167 cells (squamous cell carcinoma) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; GIBCO), 2 mM/L L-glutamine, and penicillin–streptomycin (100 U/mL and 100 mg/mL, respectively); for Daudi B cells (CCL-213; ATCC), 4.5 g/L glucose, 1.5 g/L NaHCO₃, 10 mM/L HEPES, and 1 mM/L sodium pyruvate were added.

In vitro proliferation assays

PBMC were cultured with complete medium, 15 μ M isopentenyl pyrophosphate (IPP) (Sigma) and 100 U/ml human recombinant IL-2 (Tecin, Biological Resources Branch, National Institutes of Health, Bethesda, MD, USA) in the absence or presence of rapamycin (0.05–5 nM) (Cell Signaling Technology, Inc.). Fresh medium and IL-2 were added periodically (Fig. 2). Rapamycin was added every day for the first 10 days of culture and every 3 days after 10 days thereafter. V δ 2 T cell proliferation was measured by cell counting and staining for CD3 and V δ 2. The percentage of $\gamma\delta$ T cells within the total lymphocyte population was defined by flow cytometry.

Immunoblot analysis

Cells were lysed in gel loading buffer (Invitrogen, Carlsbad, CA, USA); samples were boiled for 10 min and proteins were separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and probed with various primary antibodies. Secondary antibodies including HRP-conjugated, anti-rabbit or anti-mouse (Cell Signaling Technology, Inc.) were visualized with enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK) and exposure to Kodak X-ray film.

Cytotoxicity assay

A nonradioactive, fluorometric cytotoxicity assay [33] with calcein-acetoxymethyl (calcein-AM; Molecular Probes) was used to measure cytotoxicity against Daudi B cells Tu167 cells. $\gamma\delta$ cells (effector cells) expanded with or without rapamycin were used as effector cells. Daudi B or TU167 target cells were labeled for 15 min with 2 mM/L calcein-AM at 37°C and then washed once with PBS. Cells were combined at various effector-to-target (E:T) ratios in 96-well, round-bottomed microtiter plates (Corning, NY, USA) and incubated at 37°C in 5% CO₂ for 4 h; assays were performed in triplicate. After incubation, supernatants were transferred to a 96-well flat-bottomed microtiter plate, and calcein content was measured using a Wallac Victor2 1420 multi-channel counter (1,485/535 nm). Percent-specific lysis was calculated as: (test release-spontaneous release)/(maximum release-spontaneous release) $\times$ 100.

Flow cytometry

Unless noted, cells were stained with fluorophore-conjugated monoclonal antibodies from BD Biosciences, San Jose, CA, USA. Generally, 3×10^5 to 5×10^5 cells were washed, resuspended in 50–100 μ L of RPMI 1640, and stained with mouse anti-human V δ 2-PE/FITC clone B6, mouse anti-human CD3-FITC/APC clone UCHT1, mouse anti-human CD62L-PE clone SK11, mouse anti-human CD69-APC clone FN50, mouse anti-human CD107a-FITC clone H4A3, mouse anti-human CD80 Clone L307.4, mouse anti-human CD86 Clone 2331 (fun-1), mouse anti-human MHCII Clone Tu39 and isotype controls, including rabbit anti-mouse IgG1-FITC clone $\times$ 40, IgG1-PE clone $\times$ 40, and IgG1-APC clone $\times$ 40. For detecting intracellular IFN- γ , cells were stained with mouse anti-human V δ 2-FITC clone B6 and then fixed, permeabilized, and incubated for 45 min at 4°C with mouse anti-human IFN- γ -APC. Intracellular staining solutions were obtained from the Cytofix/Cytoperm Kit (BD Biosciences). Cells were washed with staining buffer and resuspended. Data were acquired for at least 1×10^4 lymphocytes (gated on the basis of forward- and

side-scatter profiles) from each sample, using a FACSCalibur flow cytometer (BD Biosciences). All samples were analyzed using FlowJo software (FlowJo 8.8.2, Tree Star, San Carlos, CA, USA). For stimulation before staining, V δ 2 T cells were treated with IPP for 4 h. The increase in mean fluorescence intensity (Δ MFI) was calculated as [MFI (specific mAb) – MFI (isotype control)]/MFI (isotype control).

Statistical analysis

Differences among groups were analyzed by Student's *t* test. *P* < 0.05 was considered to be significant.

Results

mTOR is part of the TCR signaling pathway in V δ 2 T cells

We first characterized the mTOR pathway in V δ 2 T cells. Freshly isolated PBMC contained 1–10% of V δ 2 T cells; after 10–14 days of culture with IPP plus IL-2, the percentage of V δ 2 T cells was >90% (Fig. 1a). V δ 2 T cell lines were washed twice and cultured in fresh medium for 24 h without stimulation. Then, cells were treated with combinations of rapamycin, U0126 (Erk inhibitor) and LY294002 (Akt inhibitor) for 1 h followed by the addition of IPP. After 20 min, cells were collected for western blotting assays. Adding IPP activated Erk and Akt as reported previously (Fig. 1b) [34]. IPP also activated S6K1 and EIF4EBP1 (Fig. 1b), two major substrates of mTORC1, indicating that mTOR was activated by TCR signaling in V δ 2 T cells. As expected, rapamycin inhibited the activation of S6K1 and EIF4EBP1, but not Erk and Akt (Fig. 1b). Inhibiting Erk (U0126) or Akt (LY294002) blocked activation of S6K1 and EIF4EBP1 (Fig. 1b). These experiments confirm that mTOR signaling is active in V δ 2 T cells, with effects similar to what has been observed in other cell types [24].

Rapamycin amplifies IPP/IL2-induced proliferation kinetics of V δ 2 T cells

We next tested the effect of rapamycin on V δ 2 T cell proliferation. PBMC were stimulated with IPP/IL2 and cultured with rapamycin (between 0.05 and 5 nM). Cultures were maintained for 44 days with periodic additions of IL2 and rapamycin. V δ 2 T cell proliferation was assessed every 3 days. Rapamycin delayed the onset of rapid proliferation among V δ 2 T cells (Fig. 2) but eventually maintained cells at higher levels for longer times compared with IPP/IL2 alone. Rapamycin treatment increased the eventual yield of V δ 2 T cells in these expansion cultures. By day 44, V δ 2 cell numbers were threefold higher with rapamycin (11 and 15 $\times 10^6$ V δ 2 T cells per ml) than without rapamycin (4 $\times 10^6$ V δ 2 T cells per ml).

Rapamycin treatment increased the eventual yield of V δ 2 T cells in our PBMC cultures. Higher doses of rapamycin (5 nM) delayed the onset of proliferation but allowed cells to reach high levels by 30 days in culture, and maintain these levels for at least 14 days. Lower doses of rapamycin maintained V δ 2 T cells at high levels after the initial burst of proliferation but did not alter the kinetics of cell growth.

V δ 2 T cells expanded with rapamycin (Rapa-V δ 2 T cell) express higher levels of CD25 or Bcl-2, and lower levels of CCR5; they have increased resistance to apoptosis

We wanted to learn more about the mechanisms for rapamycin effects on IPP/IL2-induced V δ 2 T cells. Two possible mechanisms were considered: TCR or IL2 signaling was enhanced in Rapa-V δ 2 T cells, or Rapa-V δ 2 T cells resisted apoptosis. To distinguish these effects, we started by measuring IL2 receptor (CD25) levels on V δ 2 T cells. PBMC were stimulated with IPP/IL2 in the presence or absence of increasing rapamycin doses (between 0.05 and 5 nM). On days 10 and day 30, CD25 expression was measured by flow cytometry.

Rapamycin treatment increased CD25 expression in a dose-dependent manner (Fig. 3a, e). Higher levels of receptor likely increased the sensitivity of V δ 2 T cells to IL2. The CD25 expression was maintained at higher levels throughout the 44 days in culture and may account for part of the rapamycin effect.

V δ 2 T cells expanded without rapamycin expressed high levels of CCR5; rapamycin treatment reduced CCR5 expression in a dose-dependent manner on both day 10 and day 30 (Fig. 3b, f). The chemokine receptor CCR5 is known to mediate apoptosis in T cells upon binding its ligand RANTES (Regulated upon Activation Normal T cell Expressed and Secreted, CCL5) [35]. We reported previously that rapamycin treatment reduced CCR5 mRNA levels in macaques [32]. We also know that unstimulated V δ 2 T cells contain cytoplasmic RANTES that is released immediately after antigen stimulation [36]. We do not yet know whether CCR5 levels in V δ 2 cells were modulated due to increased ligand or decreased gene expression.

Bcl-2 is an important anti-apoptotic protein [37]. We monitored the expression of Bcl-2 on days 10 and 30. Rapa-V δ 2 T cells expressed higher levels of Bcl-2 (Fig. 3c, g) that might increase their resistance to apoptosis. We tested apoptosis resistance by treating V δ 2 T cell lines with a Fas-specific antibody at 30 days after antigen exposure. Cells were collected 4 h after adding Fas antibody, then stained with antibody against AnnexinV. Rapa-V δ 2 T cells resisted anti-Fas antibody-induced apoptosis. With increasing rapamycin, AnnexinV⁺ cells decreased from $50 \pm 5.9\%$ to $20 \pm 2.7\%$ (Fig. 3d, h).

Rapa-V δ 2 T cells have a higher proportion of CD62L⁻CD69⁺ cells and stronger TCR-dependent responses

Next, we wanted to know whether rapamycin altered the expression of cell surface markers related to V δ 2 T cell activation. Cells were stimulated with IPP in the presence or absence of increasing rapamycin doses (between 0.05 and 5 nM). On day 30, cells were stained with antibodies for CD62L (marker of naïve cells) or CD69 (marker of activated cells). With increasing rapamycin, there was a decrease in CD62L⁺ cells from 49 to 14%, and a corresponding increase in the proportion of cells expressing CD69 from 36 to 69% (Fig. 4a). The addition of rapamycin stably increased the proportion of activated V δ 2 T cells in this culture system.

We measured functional responses of V δ 2 T cell lines to TCR-stimulation. Expanded V δ 2 T cells (day 30) were re-stimulated with IPP. After 4 h, IFN- γ production and CD107a expression (a marker for degranulation) were detected by flow cytometry. We found that only CD62L⁻ V δ 2 T cells responded to IPP stimulation and this population was increased after rapamycin treatment (Fig. 4b). There was a dose-dependent increase in cells expressing IFN- γ or CD107a; these markers of effector function were only seen on the CD62L⁻ subset. We also compared tumor cell cytotoxicity with and without rapamycin treatment. We used Daudi B (a Burkitt's lymphoma) and TU167 (a squamous carcinoma) cell lines as target cells. V δ 2 T cells expanded in the presence of rapamycin (5 nM, day 30) demonstrated higher cytotoxicity against both Daudi B (Fig. 4c) and TU167 cell lines (Fig. 4d) compared with cells cultured without rapamycin.

Rapa-V δ 2 T cells express higher levels of APC molecules

It was reported that activated V δ 2 T cells acquire numerous features of antigen-presenting cells (APC), such as the capacity for antigen presentation or costimulation [38]. We wanted to know whether rapamycin altered cell surface markers associated with APC function, especially the expression of costimulatory molecules. We tested the expression of MHC-II, CD80, and CD86 on days 10 and 30. With increasing rapamycin, Rapa-V δ 2 T cells

expressed higher levels of MHC-II, CD80, and CD86 molecules (Fig. 5), with the most pronounced differences seen for MHC-II.

Discussion

Rapamycin is an immunosuppressive drug for organ transplantation [19] but the mechanisms of action are complex and incompletely understood. Rapamycin affects several different types of immune cells [26]; until now, effects on V δ 2 T cells were not reported. We found that rapamycin modulated IPP-induced V δ 2 T cell proliferation and altered the phenotype of expanded cells. Appropriate doses of rapamycin increased the yield of V δ 2 T cells after antigen stimulation and altered their functional characteristics including decreased susceptibility to apoptosis and increased effector activity.

We investigated the possible mechanisms of action for rapamycin on V δ 2 T cells. Rapamycin inhibited mTORC1 and reduced the strength of TCR signaling in V δ 2 T cells, which may explain why higher dose treatment delayed the onset of rapid cell proliferation. Further, we considered four possible explanations for the rapamycin effects: first, rapamycin increased the expression of IL2 α -chain (CD25), which might increase IL2-dependent proliferation and survival. This is consistent with a previous report that rapamycin selectively expands CD4⁺CD25⁺ regulatory T cells [39] which may occur because of enhanced response to IL2. Second, V δ 2 T cells grown in the presence of rapamycin were resistant to apoptosis, possibly due to down-regulation of CCR5, up-regulation of Bcl-2, and resistance to Fas-mediated signaling. Third, rapamycin treatment increased the proportion of CD62L⁻ cells which have higher effector activity including cytokine expression and cytotoxicity. Fourth, rapamycin increased the expression of cell surface markers associated with antigen presentation.

CCR5 and its cognate ligand RANTES were identified originally for their selective chemoattractant effects [40]. However, there is accumulating evidence for CCR5-mediated apoptosis. RANTES-induced T cell death has been implicated as a potential immune escape mechanism in melanoma progression, associated with CCR5-mediated cytochrome c release and activation of caspases-9 and -3 [41]. CCR5-mediated activation of caspase-3 and cell death have been reported in neuroblastoma cells [42]. There is also evidence that apoptosis of bystander (uninfected) CD4 T cells, after exposure to HIV particles, is CCR5-dependent [43]. RANTES-mediated T cell apoptosis depended on cell-surface glycosaminoglycan binding [35]. Modulating CCR5 levels by rapamycin may reduce RANTES-mediated apoptosis of V δ 2 T cells.

Bcl-2 plays a central role in cell survival by preventing apoptosis [37]. Rapamycin increased the expression of Bcl-2 in some immune cells, including B cells [44], CD8 T cells [31], and CD4⁺CD25⁺ regulatory T cells [39]. Consistent with these studies, we found that rapamycin up-regulated the expression of Bcl-2 in V δ 2 T cells, which likely contributed to resistance against Fas-mediated apoptosis.

We also assessed the impact of rapamycin on V δ 2 T cell activation. After phosphoantigen stimulation and cell proliferation, we accumulated V δ 2 T cells that lacked expression of CD27, CD45RA, or CCR7 making it harder to distinguish familiar subsets [45]. Cells could be separated according to the pattern of CD62L and CD69 expression, including CD62L⁺CD69⁻, CD62L⁻CD69⁻, and CD62L⁻CD69⁺ subsets. We did not find appreciable levels of CD62L CD69 double-positive V δ 2 T cells. CD62L and CD69 are recognized as naïve and active markers, respectively. In our hands, effector activities after TCR-stimulation were observed only in the CD62L⁻ V δ 2 T cells. Rapamycin treatment increased

the proportion of CD62L⁺CD69⁺ cells in expanded V δ 2 T cell lines and thus, improved function by increasing the response to TCR stimulation.

One attractive feature of V δ 2 T cells is their ability to act as professional antigen-presenting cells (APC). Activated V δ 2 T cells acquire characteristics of professional APC, such as the expression of antigen presenting, costimulatory, and adhesion molecules, including MHC-II, CD80, and CD86. Rapamycin treatment increased expression of MHC-II, CD80, and CD86 on V δ 2 T cell lines. Previous studies reported that rapamycin inhibits MHC-II and costimulatory molecule expression by bone marrow-derived dendritic cells in the mouse [46]. Apparently, rapamycin has the opposite effect in V δ 2 T cells, but we did not conduct formal tests to measure their potency in antigen presentation assays.

Rapamycin increased the yield and apoptosis-resistance of V δ 2 T cells in culture. In this system, we studied functional responses to antigen re-stimulation, including cytokine production or degranulation, but cells proliferated poorly after the second exposure to antigen. This is likely due to a lack of dendritic cells in these long-term cultures which are >90% V δ 2 T cells. Thus, our in vitro model does not allow a direct test of whether rapamycin treatment can reduce the anergy to phosphoantigen, which was apparent after multiple antigen exposures in man [14] or macaques [17]. However, the increased cell yields and higher antigen responses (including increased proportion of CD62L⁺ cells) argue that rapamycin studies should be conducted in non-human primates. If we can extend the interval of elevated and functional V δ 2 T cells following phosphoantigen or bisphosphonate treatment, it may be possible to increase the therapeutic effect of immunotherapies that exploit functional properties of V δ 2 T cells.

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Fig. 1.

mTOR is involved in TCR signaling pathway of Vδ2 T cells. **a** Freshly isolated PBMC contained 1–10% of Vδ2 T cells (3.12% in *upper panel a*); after 10–14 days of culture with IPP (15 μM) plus IL-2 (100 U/ml), the percentage of Vδ2 T cells was more than 90% (92.4% in *lower panel a*). **b** Vδ2 T cell lines (corresponding to *lower panel a*) were washed twice and cultured in fresh medium for 24 h before they were treated with rapamycin, U0126 (Erk inhibitor) or LY294002 (Akt inhibitor) for 1 h followed by the addition of IPP (15 μM) for 20 min. After stimulation, cells were collected for western blotting assay with specific antibodies. Data are representative of three independent experiments with cells from different donors

Fig. 2.

Rapamycin alters the kinetics of IPP/IL2-induced Vδ2 T cell proliferation. PBMC were stimulated with IPP/IL2 and maintained in rapamycin at the doses indicated (between 0.05 and 5 nM). Cultures were maintained for 44 days with periodic addition of low-dose IL2. The proliferation kinetics of Vδ2 T cells was monitored by determining the frequency (a) and absolute cell numbers (b) of Vδ2 T cells every 3 days. Data are representative of three independent experiments with cells from different donors

Fig. 3. V δ 2 T cells expanded in the presence of rapamycin (Rapa-V δ 2 T cell) express higher levels of CD25 and Bcl-2, lower levels of CCR5 and resist apoptosis. PBMC were stimulated with IPP/IL2 and maintained in rapamycin at different doses (between 0.05 and 5 nM) as described in Fig. 2. On days 10 and 30, CD25 (**a**, **e**), CCR5 (**b**, **f**) or Bcl-2 (**c**, **g**) expression on V δ 2 T cells was measured by flow cytometry. Curves are color-coded to indicate the amount of rapamycin used or to show the isotype control staining. The increase in mean fluorescence intensity (Δ MFI) was calculated as: $[\text{MFI (specific mAb)} - \text{MFI (isotype control)}] / \text{MFI (isotype control)}$. **d**, **g** The expanded V δ 2 T cells (day 30) were stimulated with anti-Fas antibody. After 4 h, cells were collected, stained with AnnexinV and evaluated by flow cytometry. The statistical significance compared with medium control was analyzed (**e-h**). * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0001$ (Student's t test). Data are representative of three independent experiments (error bars, SD)

Fig. 4.

Rapa-V δ 2 T cells have a higher proportion of CD62L⁻CD69⁺ cells and produce stronger TCR-dependent responses. **a** PBMC were stimulated with IPP/IL2 in the presence or absence of increasing doses of rapamycin (between 0.05 and 5 nM) as described in Fig. 2. On day 30, V δ 2 T cells were stained with antibodies for CD62L (marker of naïve cells) or CD69 (marker of activated cells) and analyzed by flow cytometry. **b** The expanded V δ 2 T cells (day 30) were stimulated with IPP. After 4 h, IFN- γ production and CD107a expression were detected by flow cytometry. Data are representative of three independent experiments with cells from different donors. **c, d** The cytotoxicity of expanded V δ 2 T cell (day 30) in the presence or absence of rapamycin (5 nM) against Daudi (**c**) or TU167 (**d**) was evaluated at different E:T ratios in triplicate. The statistical significance of specific lysis compared with medium control was analyzed (Student's *t* test). **P* < 0.05. Data are representative of two independent experiments

Fig. 5.

Rapa-V δ 2 T cells express higher levels of APC molecules. PBMC were stimulated with IPP/IL2 and maintained in rapamycin at doses between 0.05 and 5 nM as described in Fig. 2. On days 10 and 30, MHC-II (a, d), CD80 (b, e) or CD86 (c, f) expression on V δ 2 T cells was measured by flow cytometry. Data are representative of three independent experiments with cells from different donors. The increase in mean fluorescence intensity (Δ MFI) was calculated as: [MFI (specific mAb) – MFI (isotype control)]/MFI (isotype control). The statistical significance compared with medium control was analyzed (d–f). * P < 0.05, ** P < 0.005, *** P < 0.0001 (Student's t test). Data are representative of three independent experiments (error bars, SD)