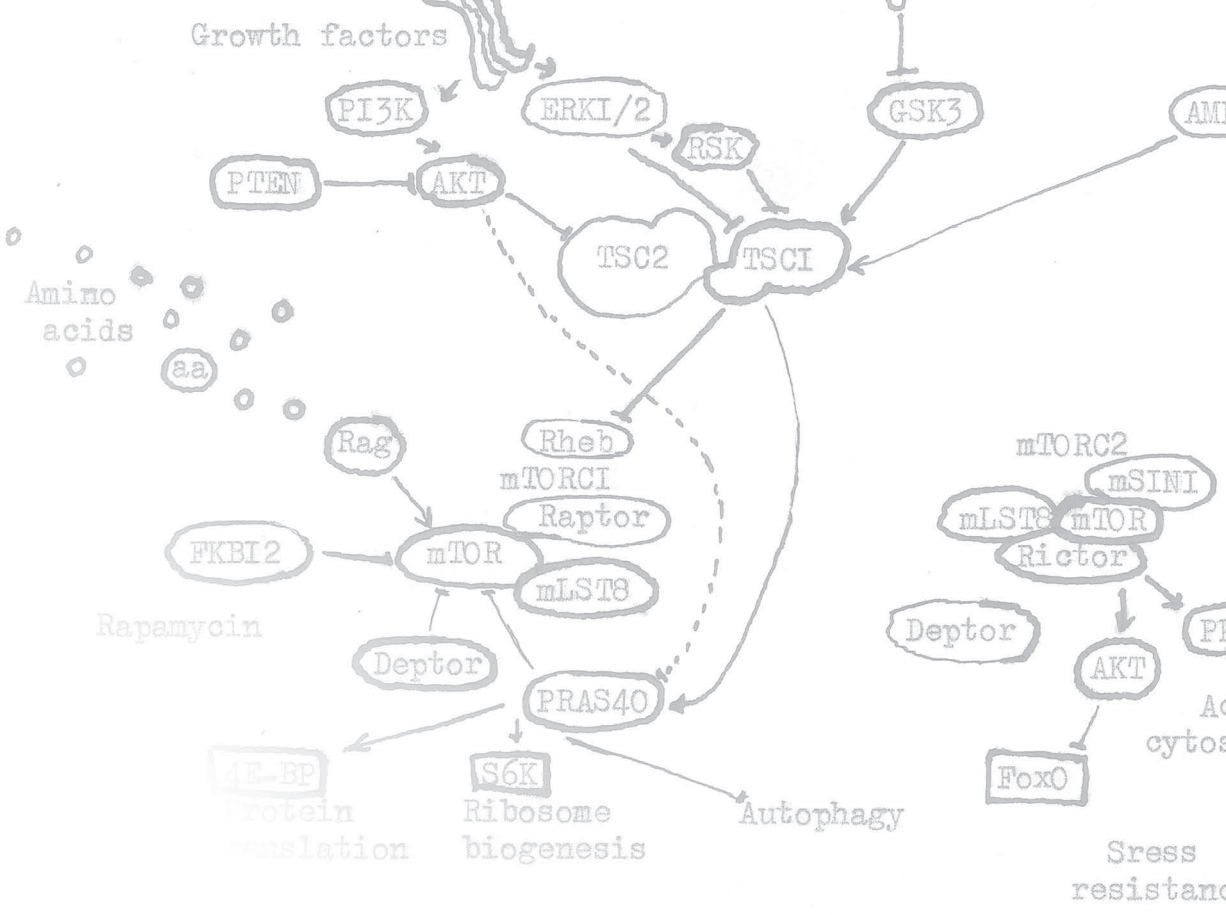

RAPAMYCIN AND OMEGA-1: mTOR-DEPENDENT AND -INDEPENDENT TH2 SKEWING BY HUMAN DENDRITIC CELLS

Leonie Hussaarts, Hermelijn H. Smits, Gabriele Schramm, Alwin J. van der Ham, Gerard C. van der Zon, Helmut Haas, Bruno Guigas and Maria Yazdanbakhsh

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ABSTRACT

Recent reports have attributed an immunoregulatory role to the mammalian target of rapamycin (mTOR), a key serine/threonine protein kinase integrating input from growth factors and nutrients to promote cell growth and differentiation. In the present study, we investigated the role of the mTOR pathway in Th2 induction by human monocyte-derived dendritic cells (moDCs). Using a co-culture system of human lipopolysaccharide (LPS)-matured moDCs and allogeneic naive $CD4^+$ T cells, we show that inhibition of mTOR by the immunosuppressive drug rapamycin reduced moDC maturation and promoted Th2 skewing. Next, we investigated whether antigens from helminth parasites, the strongest natural inducers of Th2 responses, modulate moDCs via the mTOR pathway. In contrast to rapamycin, neither *Schistosoma mansoni*-soluble egg antigens (SEA) nor its major immunomodulatory component omega-1 affected the phosphorylation of S6 kinase (S6K) and 4E-binding protein 1 (4E-BP1), downstream targets of mTORC1. Finally, we found that the effects of rapamycin and SEA/omega-1 on Th2 skewing were additive, suggesting two distinct underlying molecular mechanisms. We conclude that conditioning human moDCs to skew immune responses towards Th2 can be achieved via an mTOR-dependent and -independent pathway triggered by rapamycin and helminth antigens, respectively.

INTRODUCTION

The interface of immunology and metabolism is an emerging field that focuses on the relation between cell-intrinsic metabolic processes and the responses of immune cells (1;2). The area has received increasing attention, as it is now recognized that the behavior of the cells of the immune system is at least partially controlled by various metabolic pathways (2). Among them, the mammalian target of rapamycin (mTOR) pathway was recently shown to have an important role in dendritic cell (DC)-mediated T helper cell differentiation (3;4). mTOR is a serine/threonine protein kinase constituting the catalytic subunit of two functionally distinct multiprotein complexes, designated mTORC1 and mTORC2. mTORC1 integrates input from growth factors and nutrient sensors, upon which it interacts with its downstream targets S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) to promote or inhibit cell growth. mTORC2 mediates cytoskeletal organization, predominantly via interaction with protein kinase C family members and serum/glucocorticoid-regulated kinase 1 (5). The two complexes differ, among others, in that mTORC1 is highly sensitive to acute inhibition by the immunosuppressive drug rapamycin, while mTORC2 is not (6;7).

The role of the mTOR pathway in DC-mediated T-cell differentiation has mainly been studied in murine models. A decade ago, an early report showed that silencing of PI3K, which is located upstream of mTOR, increases DC IL-12 production and promotes protective Th1 responses against *Leishmania major* infection (8). More recent studies have demonstrated that differentiation of murine bone marrow-derived DCs with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 in the presence of rapamycin generates DCs with low levels of co-stimulatory molecules (9;10) which are weak stimulators of $CD4^+$ T cells and promote the expansion of regulatory T cells (10). In addition, upon exposure to lipopolysaccharide (LPS), rapamycin-treated bone marrow-derived DCs were shown to promote IL-4 secretion by T cells while decreasing IL-17 and IL-2 production (11), hinting towards a link between mTOR inhibition and Th2 induction.

In the present study, we therefore investigated the role of the mTOR pathway on Th2 polarization by human monocyte-derived DCs (moDCs). For this purpose, we compare the effects of rapamycin to those of *Schistosoma mansoni*-soluble egg antigens (SEA), the strongest natural inducer of Th2 responses (12), and omega-1, a single molecule recently identified to be the major SEA component involved in Th2 skewing (13-15).

RESULTS

In order to investigate the effect of mTOR inhibition on Th2 polarization by human DCs, we used a co-culture system of human LPS-matured moDCs and allogeneic naive $CD4^+$ T cells, a well-characterized model reflecting the *in vivo* polarization of immune responses against pathogens and pathogen-derived compounds (13;16). Blocking the mTOR pathway in moDCs using rapamycin prior to Toll-like receptor-4 ligation reduced the LPS-driven upregulation of the maturation markers CD40, CD80 and CD86 (Figure 1a, Supplementary Figure S1). In addition, like SEA and omega-1, rapamycin modulated moDCs to instruct T cells towards a

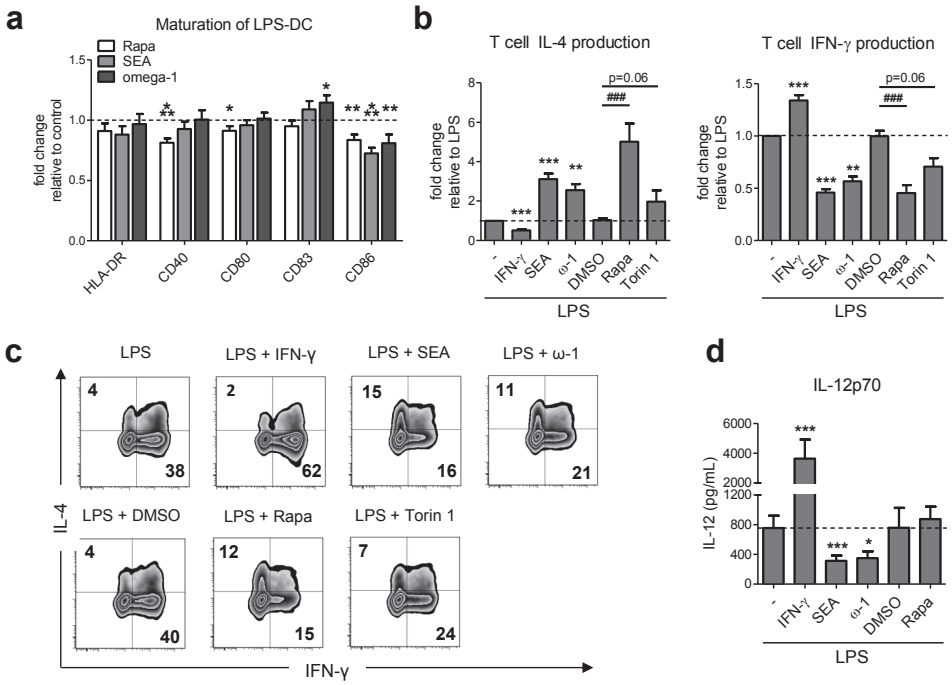

Figure 1. Rapamycin lowers DC maturation and primes for Th2 responses without affecting DC IL-12 production. (a) moDCs were either pulsed with SEA or omega-1 in the presence of LPS, or incubated for 90 min with rapamycin or its vehicle dimethyl sulfoxide (DMSO) prior to LPS stimulation. After 48 h, expression of maturation markers was analyzed by flow cytometry. The expression levels, based on the geometric mean fluorescence of at least nine independent experiments, are shown relative to the moDCs stimulated with LPS or LPS + DMSO alone, which were set to 1 for each marker (dashed line). (b) moDCs were either pulsed with SEA or omega-1 in combination with LPS, or incubated for 90 min with rapamycin, Torin1 or their vehicle DMSO prior to LPS stimulation. After 48 h of stimulation, moDCs were cultured with allogeneic naive CD4+ T cells for 11 days. Intracellular IL-4 and IFN-γ were analyzed by flow cytometry after a 6-h stimulation with phorbol myristate acetate and ionomycin. IFN-γ-stimulated moDCs were taken along as a Th1-inducing control. The percentages of T cells uniquely positive for either IL-4 or IFN-γ are expressed relative to the LPS condition. Data represent at least four independent experiments. (c) Representative FACS plots from one out of the four independent experiments are shown for conditions described for Figure 1b. (d) Following stimulation as described for Figure 1a, conditioned moDCs were co-cultured with a CD40L-expressing cell line. Supernatants were collected after 24 h and IL-12p70 concentrations were determined by ELISA. IFN-γ-stimulated moDCs were taken along as an IL-12 inducing control. Bars represent mean values + s.e.m.; *P<0.05; **P<0.01; ***, ###P<0.001 for significant differences with the control (*; either LPS or LPS + DMSO) or between test conditions (#), based on one-sided Wilcoxon-signed rank testing. ω-1, omega-1; Rapa, rapamycin.

Th2 response characterized by increased expression of IL-4 and decreased interferon (IFN)-γ production (Figures 1b and c, Supplementary Figure S2). Interestingly, in contrast to SEA and omega-1, rapamycin skewed Th2 responses without affecting the pro-inflammatory cytokine IL-12 (Figure 1d). Of note, Torin1, a second-generation highly selective inhibitor of both mTORC1 and mTORC2 (17), also induced skewing towards Th2 when added to moDCs (Figures 1b and c,

Supplementary Figure S2). However, Torin1 was a less potent Th2-inducer than rapamycin, even though blocking of mTORC1 by Torin1 and rapamycin was equally effective (data not shown). This difference in potency may reflect the complex and distinct molecular mechanisms by which the two drugs operate (18), which for rapamycin has even been described to differ between cell types (7).

As both helminth antigens and rapamycin reduce CD86 on moDCs and prime for Th2 skewing, we hypothesized the existence of a common underlying mechanism involving inhibition of mTOR activity. We therefore addressed the question whether SEA and omega-1, like rapamycin, inhibit the mTOR pathway and thereby modulate moDCs for Th2 polarization. To this end, moDCs were stimulated with LPS in the presence of helminth antigens or rapamycin for 6 and 48 h, and the phosphorylation states of mTOR and the mTORC1 downstream targets S6K and 4E-BP1 were determined. Although rapamycin strongly inhibited LPS-induced mTORC1 activity at 6 and 48 h, as shown by reduced phosphorylation of both S6K and 4E-BP1, no such effects were observed with either SEA or omega-1 (Figures 2a and b). A similar trend was observed when phosphorylation of mTOR was analyzed (Figure 2a). Interestingly, co-stimulation with rapamycin and SEA or omega-1 promoted a stronger Th2 response than stimulation with rapamycin, SEA or omega-1 alone (Figures 2c and d, Supplementary Figure S3), suggesting the existence of both mTOR-dependent and -independent mechanisms for Th2 skewing.

DISCUSSION

Recent studies have indicated that signaling pathways involved in the regulation of metabolism can control immune-cell fate and functions (2). Although much is known about the control of Th1 and Th17 differentiation, the mechanisms by which DCs skew Th2 remain largely unknown to date (19). In this study, we analyzed the role of the mTOR pathway in the induction of Th2 responses by human moDCs. To our knowledge, the present study is the first one reporting that inhibition of the mTOR pathway using the immunosuppressive drug rapamycin primes human moDCs to induce strong Th2 responses characterized by increased IL-4 and decreased IFN-γ production. This finding apparently differs from the one reported by Turnquist et al. (11), showing that rapamycin-conditioned human moDCs promote the expansion of both IL-4 and IFN-γ-producing T cells. However, it is important to note that in the study by Turnquist et al. (11), moDCs were differentiated using IL-4 and GM-CSF in the presence of rapamycin. It has been described that GM-CSF activates the mTOR pathway, and differentiation of moDCs in the presence of rapamcyin hampers the survival and the immunostimulatory capacity of moDCs (20). In contrast, in our model, moDCs were exposed to rapamycin after differentiation. Our findings are therefore complementary to the study by Turnquist et al. (11), and highlight that the effect of rapamycin on moDC-mediated T-cell polarization critically depends on the timing and the duration of exposure to rapamycin.

In addition to the effect of rapamycin on T-cell skewing, we found that exposure to the drug reduces moDC maturation. These effects are similar to those induced by the helminth

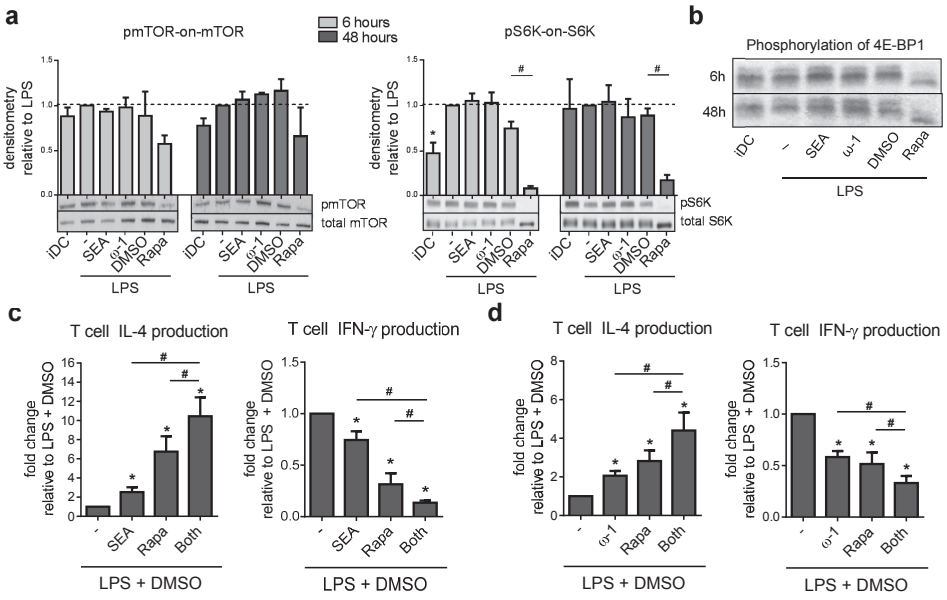

Figure 2. Unlike rapamycin, SEA and omega-1 do not affect the mTOR pathway to condition DCs for Th2 priming. MoDCs were stimulated as described in the legend of Figure 1. (a,b) moDC lysates were collected after 6 and 48 h of stimulation. Proteins were visualized by western blotting using antibodies against S6K and S6K phosphorylation at Thr421, and mTOR and mTOR phosphorylation at Ser2448. Western blot results were quantified using ImageJ software. The pS6K-on-S6K and pmTOR-on-mTOR ratios were calculated relative to the LPS condition. Representative blots from one out of five (6 h) or four (48 h) independent experiments are shown. (b) moDC lysates were collected after 6 and 48 h of stimulation. 4E-BP1 phosphorylation at Thr37/46 was assessed by western blotting. (c,d) moDCs stimulated for 48 h were cultured with allogeneic naive CD4+ T cells for 11 days and T-cell cytokine production was analyzed as described in the legend of Figure 1. Data represent at least five independent experiments. Bars represent mean values + s.e.m.; *,#P<0.05 for significant differences with the control (*; either LPS or LPS + DMSO) or between test conditions (#) based on one-sided Wilcoxon-signed rank testing. iDC, immature (nonstimulated) moDC; ω-1, omega-1; Rapa, rapamycin; Both, rapamycin and SEA or omega-1.

antigens, SEA and omega-1, which suppress DC maturation and are the strongest natural inducers of Th2 responses (12;13;15). Importantly, we demonstrate that SEA and omega-1, unlike rapamycin, do not affect the phosphorylation of mTOR and the mTORC1 downstream targets S6K and 4E-BP1. The finding that rapamycin and helminth antigens affect moDCs in a distinct manner is further supported by the differential effect of rapamycin and SEA or omega-1 on moDC IL-12 production.

Although both SEA and omega-1 reduce IL-12 levels, as reported previously (13), rapamycin does not. Secretion of IL-12 by antigen-presenting cells has classically been associated with Th1 differentiation (21), and the mere absence of IL-12 and other Th1-inducing molecules was suggested to promote a Th2 response (22). As rapamycin, unlike SEA and omega-1, does not attenuate moDC IL-12 secretion, this so-called default hypothesis for Th2 polarization fails to

explain the dominant Th2 response induced by rapamycin. Our results suggest the existence of mTOR-dependent and -independent mechanisms for Th2 skewing, which is further supported by experiments that show an additive effect on Th2 priming by moDCs co-stimulated with both rapamycin and helminth antigens. Needless to say, we do not exclude the possibility that rapamycin and helminth antigens target other common downstream pathways. Whether rapamycin may have similar effects on T-cell polarization by different DC subsets, remains an intriguing area of research, as it has been demonstrated that rapamycin differentially affects moDCs and myeloid DCs (20).

In summary, our observations suggest that conditioning human DCs to skew immune responses towards Th2 can be achieved by an mTOR-dependent and an mTOR-independent mechanism by rapamycin and helminth antigens, respectively. These findings are important when considering the increasing interest in DC-based immune therapy for cancer (23), transplantation and autoimmunity (24). This requires in-depth understanding of the different modes and mechanisms, whereby DCs can be accurately modulated for clinical use.

METHODS

Preparation and purification of *S. mansoni*-derived antigens

SEA and omega-1 from *S. mansoni* were prepared as described previously (25;26). The purity of the preparations was controlled by SDS-PAGE and silver staining. The protein concentrations were determined using the Bradford or BCA procedure.

Human DC culture, stimulation and analysis

Peripheral blood mononuclear cells were isolated from venous blood of healthy volunteers diluted in an equivalent volume of HBSS, by means of a Ficoll density gradient. Monocytes were isolated by positive magnetic cell sorting using CD14-beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured in RPMI supplemented with 10% FCS, human rGM-CSF (20 ng/ml; BioSource/Invitrogen, Carlsbad, CA, USA) and human rIL-4 (0.86 ng/mL, R&D Systems, Minneapolis, MN, USA). The culture medium including supplements was replaced on day 2 or 3. Immature moDCs were stimulated on day 6. Indicated conditions were preincubated with rapamycin (100 nM; Sigma-Aldrich, Zwijndrecht, The Netherlands) or Torin1 (100 nM; a kind gift from Dr. Thomas Weichhart) or their vehicle dimethyl sulfoxide for 90min prior to stimulation with ultrapure LPS (100 ng/mL; *Escherichia coli* 0111 B4 strain, InvivoGen, San Diego, CA, USA) supplemented with human rGM-CSF. Alternatively, moDCs were pulsed with SEA (50 µg/mL), omega-1 (500 ng/mL) or IFN-γ (1000 U/mL) in the presence of ultrapure LPS (100 ng/mL) and human rGM-CSF. After 48 h of stimulation, surface expression of co-stimulatory molecules was determined by FACS (FACS- Canto, BD Biosciences, Breda, The Netherlands) using the following antibodies: CD86 FITC, CD40 APC, CD80 Horizon V450 (all BD Biosciences), HLA-DR APC-eFluor 780 (eBioscience, San Diego, CA, USA) and CD83 PE (Beckman-Coulter, Fullerton, CA, USA). Only live cells, which were negative for 7AAD (eBioscience), were included in the analysis. In addition, $1x10^4$ matured moDCs were co-

cultured with $1x10^4$ CD40L-expressing J558 cells. Supernatants were collected after 24 h and IL-12p70 concentrations were determined by ELISA using mouse anti-human IL-12 (clone 20C2) as a capture antibody and biotinylated mouse-anti-human IL-12 (clone C8.6) as a detection antibody (BD Biosciences).

Human T-cell culture and analysis of T-cell polarization

For analysis of T-cell polarization, 48-h-pulsed moDCs were cultured with allogeneic naive $CD4^+$ T cells for 11 days in the presence of staphylococcal enterotoxin B (10 pg/mL). On days 6 and 8, rhuIL-2 (10 U/mL, R&D Systems) was added and the T cells were expanded until day 11. Intracellular cytokine production was analyzed after restimulation with 100 ng/mL phorbol myristate acetate plus 2 µg/mL ionomycin for 6 h; 10 µg/ml brefeldin A was added during the last 4 h and the cells were fixed with 3.7% paraformaldehyde (all Sigma-Aldrich). The cells were permeabilized with 0.2% saponin (Sigma-Aldrich) and stained with PE- and FITC-labelled antibodies against IL-4 and IFN-γ, respectively, (BD Biosciences).

Western blot analysis

moDC lysates were collected after 6 and 48 h of stimulation. Western blotting was performed as described previously,(27) the primary antibodies used were: mTOR; mTOR-Ser2448; S6K-Thr421; p4E-BP1-Thr37/46 (Cell Signaling Technology, Danvers, MA, USA) and S6K (Santa Cruz Biotechnology, Dallas, TX, USA). Western blot results were quantified using ImageJ software (NIH, Bethesda, MD, USA) and the phosphorylations of S6K (pS6K) and mTOR (pmTOR) were expressed relative to their total proteins as the pS6K-on-S6K ratio and the pmTOR-on-mTOR ratio, respectively.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 (GraphPad Software, La Jolla, CA, USA) for Windows.

ACKNOWLEDGEMENTS

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SUPPLEMENTAL DATA

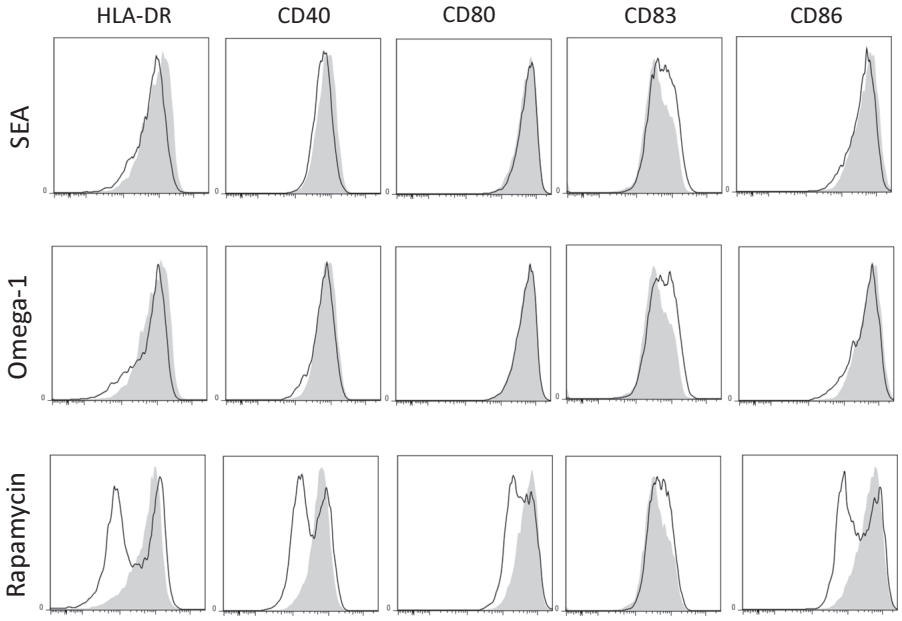

Figure S1. Rapamycin lowers LPS-induced DC maturation. moDCs were either pulsed with SEA or omega-1 in the presence of LPS, or incubated for 90 minutes with rapamycin or its vehicle DMSO prior to LPS stimulation. After 48 hours, expression of maturation markers was analyzed by flow cytometry. Representative histograms from 1 out of 9 independent experiments are shown. Filled histograms represent control conditions (either LPS or LPS + DMSO).

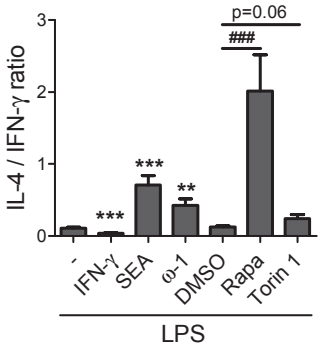

Figure S2. Rapamycin primes DCs for Th2 responses. moDCs were either pulsed with SEA or omega-1 in combination with LPS, or incubated for 90 minutes with rapamycin, Torin1 or their vehicle DMSO prior to LPS stimulation. After 48 hours of stimulation, moDCs were cultured with allogeneic naive $CD4^+$ T cells for 11 days. Intracellular IL-4 and IFN-γ were analyzed by flow cytometry after 6h stimulation with PMA and ionomycin. IFN-γ-stimulated moDCs were taken along as a Th1-inducing control. The ratio of T cells uniquely positive for either IL-4 or IFN-γ was calculated. Data represent at least 4 independent experiments. Bars represent mean values + s.e.m; ***P* <0.01; ***, ### *P*<0.001 for significant differences with the LPS control (*) or between test conditions (#) based on one-sided Wilcoxon signed rank testing. ω-1, omega-1; Rapa, rapamycin.

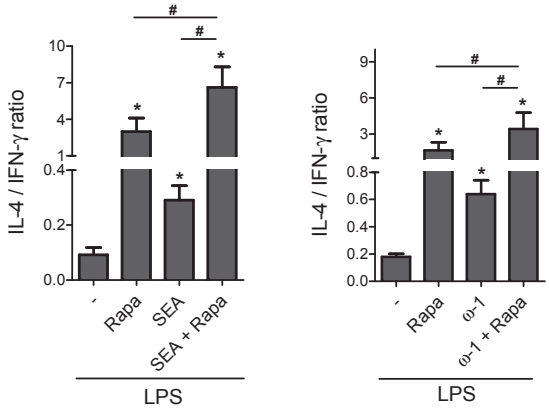

Figure S3. Co-stimulation with rapamycin and SEA or omega-1 primes a stronger Th2 response than stimulation with rapamycin, SEA or omega-1 alone. moDCs were either pulsed with SEA or omega-1 in combination with LPS, and / or incubated for 90 minutes with rapamycin, or its vehicle DMSO prior to stimulation. T cell responses were analysed as described for Supplementary figure 2. Bars represent mean values + s.e.m.; *,# *P* <0.05; for significant differences with the LPS control (*) or between test conditions (#) based on one-sided Wilcoxon signed rank testing. ω-1, omega-1; Rapa, rapamycin.