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***n* – 3 polyunsaturated fatty acids suppress phosphatidylinositol 4,5-bisphosphate-dependent actin remodelling during CD4⁺ T-cell activation**

Tim Y. Hou^{*,†}, Jennifer M. Monk^{†,‡}, Yang-Yi Fan^{†,‡}, Rola Barhoumi^{§,||}, Yong Q. Chen[¶], Gonzalo M. Rivera^{**}, David N. McMURRAY^{†,‡,§,||,††,§§}, and Robert S. Chapkin^{*,†,‡,§,||,††,1}

^{*}Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas, U.S.A.

[†]Program in Integrative Nutrition and Complex Diseases, Texas A&M University, College Station, Texas, U.S.A.

[‡]Texas AgriLife Vegetable Crop Improvement Center, Texas A&M University, College Station, Texas, U.S.A.

[§]Center for Environmental and Rural Health, Texas A&M University, College Station, Texas, U.S.A.

^{||}Department of Veterinary Integrative Biosciences, Texas A&M University, College Station, Texas, U.S.A.

[¶]Department of Cancer Biology, Wake Forest University, Winston-Salem, NC, U.S.A.

^{**}Department of Veterinary Pathobiology, Texas A&M University, College Station, Texas, U.S.A.

^{††}Faculty of Nutrition, Texas A&M University, College Station, Texas, U.S.A.

^{§§}Department of Microbial and Molecular Pathogenesis, Texas A&M University System Health Science Center, Texas A&M University, College Station, Texas, U.S.A.

Abstract

n – 3 PUFA (polyunsaturated fatty acids), i.e. DHA (docosahexaenoic acid), found in fish oil, exhibit anti-inflammatory properties; however, the molecular mechanisms remain unclear. Since PtdIns(4,5)*P*₂ resides in raft domains and DHA can alter the size of rafts, we hypothesized that PtdIns(4,5)*P*₂ and downstream actin remodelling are perturbed by the incorporation of *n* – 3 PUFA into membranes, resulting in suppressed T-cell activation. CD4⁺ T-cells isolated from *Fat-1* transgenic mice (membranes enriched in *n* – 3 PUFA) exhibited a 50% decrease in PtdIns(4,5)*P*₂. Upon activation by plate-bound anti-CD3/anti-CD28 or PMA/ionomycin, *Fat-1* CD4⁺ T-cells failed to metabolize PtdIns(4,5)*P*₂. Furthermore, actin remodelling failed to initiate in *Fat-1* CD4⁺ T-cells upon stimulation; however, the defect was reversed by incubation with exogenous PtdIns(4,5)*P*₂. When *Fat-1* CD4⁺ T-cells were stimulated with anti-CD3/anti-CD28-coated beads, WASP (Wiskott–Aldrich syndrome protein) failed to translocate to the immunological synapse. The suppressive phenotype, consisting of defects in PtdIns(4,5)*P*₂ metabolism and actin

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¹To whom correspondence should be addressed (r-chapkin@tamu.edu).

AUTHOR CONTRIBUTION

Tim Hou, Yang-Yi Fan, David McMurray and Robert Chapkin designed the research. Tim Hou and Jennifer Monk conducted the research. Tim Hou and Robert Chapkin analysed the data. Yong Chen carried out the MS experiments. Tim Hou and Rola Barhoumi captured the immunofluorescence images, and Gonzalo Rivera provided analytical tools for actin remodelling. Tim Hou, Jennifer Monk, David McMurray and Robert Chapkin wrote the paper. All authors participated in the editing of the paper prior to submission.

remodelling, were recapitulated in CD4⁺ T-cells isolated from mice fed on a 4% DHA triacylglycerol-enriched diet. Collectively, these data demonstrate that *n* – 3 PUFA, such as DHA, alter PtdIns(4,5)*P*₂ in CD4⁺ T-cells, thereby suppressing the recruitment of WASP to the immunological synapse, and impairing actin remodelling in CD4⁺ T-cells.

Keywords

actin remodelling; immunological synapse; *n* – 3 polyunsaturated fatty acid; PtdIns(4,5)*P*₂; T-cell activation; Wiskott–Aldrich syndrome protein

INTRODUCTION

Autoimmune diseases, such as inflammatory bowel disease (i.e. Crohn's disease and ulcerative colitis), can be attributed in part to an imbalance between T-cell activation and suppression [1]. Dietary fish oil enriched in *n* – 3 PUFA (polyunsaturated fatty acids) has been shown through clinical and epidemiological studies to have anti-inflammatory properties that are beneficial in chronic inflammatory conditions [2-5]. Specifically, the long-chain *n* – 3 PUFA, such as DHA (docosahexaenoic acid, 22:6^{4,7,10,13,16,19}) and EPA (eicosapentaenoic acid, 20:5^{5,8,11,14,17}), are thought to be the bioactive components of fish oil that exert anti-inflammatory effects. Some proposed mechanisms by which *n* – 3 PUFA suppress T-cell activation include modulation of (i) prostaglandin metabolism, (ii) nuclear transcription factors such as NF- κ B (nuclear factor κ B) and (iii) plasma membrane microdomains [3,6,7].

Lipid rafts in the plasma membrane are defined as detergent-resistant membrane fractions that are highly enriched in cholesterol and sphingolipids. These domains reside in the Lo (liquid-ordered) phase [8], and are thought to be critical in CD4⁺ T-cell activation, where the interaction between MHC II and the TCR (T-cell receptor) on the surface of the APC (antigen-presenting cell) and the T-cell respectively results in the major reorganization of the nanoscale lipid rafts, forming the IS (immunological synapse) [9-11]. The reconfiguration of lipid rafts to form the IS involves changes to lipid–lipid interactions in the membranes; specifically, Lo lipids, such as cholesterol and sphingolipids, accumulate at the IS [12,13]. The importance of lipid-lipid interactions at the IS is highlighted by studies demonstrating that dietary *n* – 3 PUFA, such as EPA, can alter the coalescence of lipid rafts to the IS [12,14].

At the IS, tyrosine kinases Lck (lymphocyte-specific protein tyrosine kinase) and ZAP-70 [ζ -chain (TCR)-associated protein kinase of 70 kDa] are activated and subsequently phosphorylate the adaptor protein LAT (linker for activation of T-cells), leading to the assembly of the signalsome comprising GADS [Grb2 (growth-factor-receptor-bound protein 2)-related adaptor protein], SLP76 [SH2 (Src homology 2) domain-containing leucocyte protein of 76 kDa], NCK (non-catalytic region of tyrosine kinase adaptor protein), ITK (interleukin-2-inducible T-cell kinase), VAV1 (VAV1 guanine-nucleotide-exchange factor), PAK (p21-activated kinase) and PLC γ 1 (phospholipase C γ 1) [15]. The proper formation of the IS, required for sustained T-cell activation, is stabilized by the actin cytoskeleton [10,16,17]. For example, Wiskott–Aldrich syndrome, characterized partly by deficient T-cell activation [18], is attributed to mutations in the gene encoding WASP (Wiskott–Aldrich syndrome protein), which regulates *de novo* nucleation of actin filaments [19]. Additionally, to emphasize the importance of the actin cytoskeleton to T-cell activation, many actin-regulating proteins, such as ERM (ezrin, radixin and moesin) proteins, talin, WAVE (WASP verprolin homologous) and ADF (actin-depolymerizing factor)/cofilin, are known to be localized at the IS [17,20-22].

Many of the aforementioned actin-remodelling proteins, including WASP, are regulated by PtdIns(4,5) P_2 [23]. Both the magnitude and the kinetics of PtdIns(4,5) P_2 metabolism are important in actin remodelling, as demonstrated during (i) macrophage phagocytosis [24], (ii) release of ERM proteins from the plasma membrane of T-cells after chemokine stimulation [25] and (iii) release and activation of cofilin in carcinoma cells upon EGF (epidermal growth factor) stimulation [26]. Since PtdIns(4,5) P_2 co-localizes in raft domains in T-cells [27], and $n-3$ PUFA such as DHA can increase the size of lipid rafts [28], we tested the hypothesis that PtdIns(4,5) P_2 is perturbed by the presence of $n-3$ PUFA, leading to a suppression of downstream actin remodelling.

By using the *Fat-1* transgenic mouse model, in which animals express an $n-3$ fatty acid desaturase cloned from *Caenorhabditis elegans*, thereby generating $n-3$ PUFA *de novo* and enriching the membrane with $n-3$ PUFA [29], we demonstrate for the first time that PtdIns(4,5) P_2 is decreased in the presence of $n-3$ PUFA in unstimulated CD4⁺ T-cells, leading to defective PtdIns(4,5) P_2 metabolism upon anti-CD3/anti-CD28 or PMA/ionomycin stimulation. The suppression of PtdIns(4,5) P_2 metabolism was associated with a defect in downstream actin remodelling in *Fat-1* CD4⁺ T-cells. These phenotypes were also seen in a dietary DHA intervention strategy. Furthermore, when *Fat-1* CD4⁺ T-cells were stimulated with anti-CD3/anti-CD28-coated beads, WASP failed to translocate to the IS. The defect in actin remodelling seen in *Fat-1* CD4⁺ T-cells was rescued by incubation with exogenous PtdIns(4,5) P_2 in a dose-dependent manner. Collectively, these results demonstrate for the first time that $n-3$ PUFA modulate a critical lipid mediator, PtdIns(4,5) P_2 , leading to downstream suppression of actin remodelling upon T-cell activation. These data extend our previous findings that early cytoskeleton-dependent T-cell activation events, such as mitochondrial translocation and Ca²⁺ signalling, are suppressed in $n-3$ PUFA-enriched CD4⁺ T-cells [30].

EXPERIMENTAL

Animals and CD4⁺ T-cell isolation

All animal protocols have been approved by the Institutional Animal Care and Use Committee at Texas A&M University and follow guidelines approved by the U.S. Public Health Service. The generation, genotyping and phenotyping of *Fat-1* transgenic mice on a C57BL/6 background have been described previously [30-32]. *Fat-1* and wild-type littermate controls were fed on a 10% safflower diet enriched in $n-6$ PUFA (Research Diets), provided *ad libitum* in a 12 h light/12 h dark cycle.

C57BL/6 mice (4–6-week-old) were fed on either an $n-6$ PUFA-enriched 5% corn oil diet or a 4% DHA ($n-3$ PUFA) triacylglycerol-enriched diet. Diets differed only in their oil composition, either (control) 5% corn oil by weight or a mixture of 57% pure DHA triacylglycerol (Martek) and corn oil [4:1 (w/w)]. Additional diet components were 20 g of casein/100 g, 41.9 g of sucrose/100 g, 22 g of corn starch/100 g, 6 g of cellulose/100 g, 3.5 g of AIN-76 mineral mix/100 g, 1 g of AIN-76 vitamin mix/100 g, 0.3 g of DL-methionine/100 g, 0.2 g of choline chloride/100 g and 5 g of dietary oil/100 g [13,31,33]. Mice were fed *ad libitum* for 3–4 weeks in a 12 h light/12 h dark cycle and the diet was changed daily to prevent the formation of oxidative by-products.

Spleens were removed aseptically and CD4⁺ T-cells were isolated by positive selection using magnetic CD4 (L3T4) microbeads according to the manufacturer's protocol (Miltenyi Biotec). CD4⁺ T-cells were resuspended in complete RPMI 1640 medium composed of 93% RPMI 1640 (containing 25 mM Hepes, Irvine Scientific), 5% heat-inactivated FBS (fetal bovine serum, Irvine Scientific), 1% GlutaMAX™ (Gibco) and 1% penicillin/streptomycin (Gibco), henceforth referred to as 'complete medium', at assay-specific concentrations [$5 \times$

10^5 cells/ml for basal PtdIns(4,5) P_2 measurements using a PtdIns(4,5) P_2 mass kit from Echelon Biosciences, 5×10^5 cells/100 μ l for T-cell activation, 3×10^6 cells/ml for immunofluorescence and 1.5×10^7 cells/250 μ l for immunoisolation).

CD4⁺ T-cell stimulation time course

Flat-bottom plates (96-well) containing plate-bound anti-CD3 (0.2 μ g/ml, eBiosciences) and anti-CD28 (1 μ g/ml, eBiosciences) were incubated overnight at 4°C. The wells were washed with PBS and 200 μ l of complete medium was added. The plates were then placed at 37°C, 5% CO₂ for at least 1 h before seeding CD4⁺ T-cells [34]. For PMA/ionomycin stimulation, 0.5 μ g/ml PMA (Sigma) and 500 nM ionomycin (EMD Chemicals) in complete medium were added into each well and incubated at 37°C, 5% CO₂ for at least 1 h before seeding T-cells [32]. CD4⁺ T-cells were seeded into each well, stimulated for specified times (0–30 min), and then transferred into low-retention 1.5 ml microcentrifuge tubes and immediately placed on ice to quench cell activation. CD4⁺ T-cells were centrifuged at 4°C at 4000 g for 5 min, and washed in ice-cold 1×PBS before lipid extraction.

Extraction of PtdIns(4,5) P_2 for detection using a PtdIns(4,5) P_2 mass kit or anti-PtdIns(4,5) P_2 ELISA

Extraction of PtdIns(4,5) P_2 has been described previously, with some modifications [35]. Briefly, after washing with ice-cold 1×PBS, the cells were pelleted at 4000 g for 5 min at 4°C (described above), the supernatant was removed and the pellet was resuspended in 800 μ l of 1:1 (v/v) methanol/chloroform. The mixture was vortex-mixed for 1 min, and centrifuged at 7500 g for 5 min at 4°C. The supernatant was removed, and the pellet was resuspended in 400 μ l of 80:40:0.3 (by vol.) methanol/chloroform/hydrochloric acid. The mixture was vortex-mixed for 5 min and subsequently centrifuged at 3000 g for 1 min at 4°C. An additional 80 μ l of 1 M HCl was added to the extract and vortex-mixed for 15 s before centrifugation at 18000 g for 15 s at 4°C. The organic layer was collected and dried under a stream of nitrogen gas. The lipid film was dissolved in 1×PBS supplemented with 0.0025% protein stabilizer (Echelon Biosciences) and was used for the detection of PtdIns(4,5) P_2 .

Detection of PtdIns(4,5) P_2

For basal level PtdIns(4,5) P_2 detection, the PtdIns(4,5) P_2 mass kit (Echelon Biosciences) was used according to the manufacturer's protocol. Briefly, the mass kit utilizes a PtdIns(4,5) P_2 protein detector that is added to a PtdIns(4,5) P_2 -coated strip for competitive binding. The colorimetric signal is inversely proportional to the amount of PtdIns(4,5) P_2 .

For determination of the CD4⁺ T-cell stimulation time course, an indirect ELISA was developed to detect the level of PtdIns(4,5) P_2 . PtdIns(4,5) P_2 dissolved in 1×PBS supplemented with 0.0025% protein stabilizer was added in duplicate into 96-well flat-bottomed plates and incubated at room temperature (23 °C) for 2 h. The wells were then washed three times with 1×PBS, and then blocked in 5% BSA in PBS (blocking solution) overnight at 4°C. The wells were again washed three times with 1×PBS, and incubated with primary mouse anti-PtdIns(4,5) P_2 (Abcam) in blocking solution at a dilution of 1:2500 for 1.5 h at room temperature. The wells were washed with 1×PBS and incubated with secondary goat anti-mouse IgG labelled with horseradish peroxidase (KPL) in blocking solution at a dilution of 1:5000 for 1 h at room temperature, protected from light. The wells were washed four times with 1×PBS, and incubated in TMB (3,3',5,5'-tetramethylbenzidine) high-sensitivity substrate solution (BioLegend) for 5 min at room temperature, protected from light. The reaction was stopped by the addition of 0.5 M sulfuric acid, and the absorbance was read at 450 nm. A standard curve was generated with known concentrations of PtdIns(4,5) P_2 (Echelon Biosciences), and was used to quantify the PtdIns(4,5) P_2 levels.

To test the cross-reactivity of the primary antibody, 50 pmol of PtdIns4*P* (Avanti) and PtdIns(3,4,5)*P*₃ (Avanti) were dissolved in 1×PBS supplemented with 0.0025% protein stabilizer, and added into separate wells and subjected to the same ELISA protocol as described above. No detectable signals were observed.

Extraction and detection of PtdIns(4,5)*P*₂ using MS

Purified splenic CD4⁺ T-cells were isolated and washed as described above. For MS, cells from two animals were pooled to increase the detection of PtdIns(4,5)*P*₂. After washing, purified splenic CD4⁺ T-cells were dissolved in a solvent composed of chloroform/methanol/water (32.6:65.3:2.1, by vol.) and extracted as described previously [36]. Dipalmitoyl-PtdIns(4,5)*P*₂ (200 ng) was added into each sample as an internal standard prior to extraction. Lipid extracts were evaporated under argon gas and redissolved in 50 μl of chloroform/methanol/water (5:5:1, by vol) and analysed by LC-MS/MS (liquid chromatography tandem MS). MS (Thermo TSQ Quantum Discovery Max) operated in the negative-ion mode was optimized to detect phosphoinositides as [*M* – H][–] ions. Using the peak areas, the mass of individual phosphoinositides was estimated and corrected by using the internal standard. Samples were normalized to cell number as determined using a Coulter Counter (Beckman Coulter).

Immunofluorescence

Immunofluorescence was conducted as described previously [31,32]. Briefly, 4.0 cm² per well chamber cover slides (Nalge) were pre-coated with 0.01% poly-L-lysine (Sigma) for 30 min. The solution was removed and the slides were allowed to air dry for 1 h at room temperature. For T-cell stimulation, chamber cover slides were additionally coated with anti-CD3 and anti-CD28 at a concentration of 1 μg/ml and 5 μg/ml respectively. The unstimulated (control) slides were incubated with 1×PBS overnight. Following CD4⁺ T-cell isolation, 3×10⁶ cells in complete medium were seeded into chamber slides and incubated for 30 min at 37 °C and 5% CO₂. For bead stimulation, 3×10⁶ CD4⁺ T-cells were incubated in the presence of Dynabeads Mouse T-activator CD3/CD28 (Invitrogen) at a 1:1 ratio for 30 min at 37 °C and 5% CO₂. For the exogenous PtdIns(4,5)*P*₂ rescue experiment, 3×10⁶ *Fat-1* CD4⁺ T-cells were incubated in the presence of exogenous PtdIns(4,5)*P*₂ at specific concentrations (Echelon Biosciences) for 1 h at 37°C and 5% CO₂ and washed once with warm 1×PBS before stimulation, as described above using plated anti-CD3/anti-CD28.

Cells were washed three times with PBS and immediately fixed in 4% paraformaldehyde for 20 min at room temperature. The chamber slides were washed with PBS and incubated in 10 mM glycine for 10 min at room temperature, followed by permeabilization using 0.2% Triton X-100 for 5 min at room temperature. The chamber slides were subsequently washed with PBS, followed by overnight incubation at 4°C in a humid chamber with blocking solution (1% IgG-free BSA, 0.1% sodium azide and 99% PBS). The slides were washed with PBS before the addition of 200 μl of 20 units/ml Alexa Fluor® 568-conjugated phalloidin (Invitrogen), incubated for 1 h in a humid chamber at room temperature, and then washed three times with PBS, and incubated in 70% ethanol, 95% ethanol, 100% ethanol and fresh xylene, for 2 min each. ProLong anti-fade medium (Invitrogen) was applied to each coverslip, and the slides were allowed to dry in the dark overnight at room temperature. Slides were sealed with fingernail polish before examination using microscopy.

For bead-stimulated samples, slides were fixed, washed, treated and blocked overnight as described above. Next, 200 μl of 20 units/ml Alexa Fluor® 568-conjugated phalloidin and 4 μg/ml rabbit anti-WASP (Santa Cruz Biotechnology) was applied to slides for 1 h in a humid chamber at room temperature before the slides were washed twice with PBS. The addition of 6 μg/ml Alexa Fluor® 647-conjugated goat anti-rabbit antibody (Invitrogen) to

slides for 1 h in a humid chamber at room temperature preceded washes and treatment as described above.

Immunoisolation

Immunoisolation was conducted as described previously, with minor modifications [37]. Briefly, 1.5×10^7 CD4⁺ T-cells were incubated with anti-CD3/anti-CD28-coated Dynabeads (Invitrogen) at a cell-to-bead ratio of 2:1 on ice for 7 min before stimulation at 37°C for 30 min. Samples were then subjected to nitrogen cavitation at 800 psi (1 psi = 6.9 kPa) for 7 min, transferred to 1.5 ml microcentrifuge tubes, = and washed three times with 1 ml of complete homogenization buffer [250 mM sucrose, 10 mM sodium Hepes, 2 mM MgCl₂, 10 mM NaF, 100 μ M sodium orthovanadate and 40 μ l/ml protease inhibitors]. Input samples (100 μ l) were assayed prior to magnetic bead collection to determine the yield of the cavitation using Western blot analysis. Samples (12 μ l) were analysed using Western blotting (see the Supplementary Experimental section at <http://www.BiochemJ.org/bj/443/bj4430027add.htm>). The enrichment factor was calculated by dividing the intensity of the band in the IS by the intensity of the band in the input for WASP.

Microscopy and image processing

Slides were examined using a Zeiss 510 META NLO laser-scanning microscope equipped with argon laser, LSD software and a $\times 63$ objective. Images were exported into TIFF format using AxioVision LE 4.8 and imported into NIS-Elements AR 3.10 (Nikon). To correct for background, a rectangular ROI (region of interest) was drawn in an area without any cells. This area was used to subtract the background from the image. ROIs were then drawn around cells that were not in contact with other cells, and the mean intensity was recorded. This value was then divided by the number of cells in the field measured to obtain the average mean intensity per cell.

For bead-stimulated samples, slides were examined using a Nikon Ti-E inverted microscope equipped with X-cite 120 fluorescence microscopy illuminator and $\times 60$ objective, with 1.5 magnification. Images were captured using a Photometrics Cool-SNAP EZ CCD (charge-coupled device) camera and analysed in NIS-Elements AR. Two-dimensional fast deconvolution was applied to all captured images. An ROI was drawn around the cell excluding the beads, and the total intensity was recorded. This value was divided by the area of the cell to obtain the average mean intensity per square micron. For the IS, the ROI was drawn as an oval at the T-cell proximal to the bead, as described previously [32]. The total intensity was recorded and divided by the area of the IS. There were no differences between the size of the cell or the IS between genotypes (results not shown); furthermore, the area of the IS was approximately $10 \pm 5\%$ of the whole cell in both genotypes (results not shown).

Statistical analysis

Values are expressed as means $\pm$ S.E.M. unless otherwise noted and analysed using SAS 9.2 for Windows. The time course was tested by two-way ANOVA (main effects, genotype and time). In cases where the interaction between genotype and time was insignificant, a least significant difference post-hoc test was conducted. In the cases where the interactions between genotype and time were significant, a post-hoc Tukey's Studentized Range test was conducted. Differences between genotypes in single treatment were analysed by two-tailed Student's *t* test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Basal PtdIns(4,5) P_2 concentration is reduced in *Fat-1* CD4⁺ T-cells

Since PtdIns(4,5) P_2 is one of the key mediators of actin remodelling, we determined whether the level of PtdIns(4,5) P_2 is perturbed in *Fat-1* CD4⁺ T-cells. Total lipid analysis revealed enrichment of *n* – 3 PUFA in *Fat-1* CD4⁺ T-cells as compared with wild-type (Supplementary Figure S1 at <http://www.BiochemJ.org/bj/443/bj4430027add.htm>). We first used MS to determine both total PtdIns(4,5) P_2 mass and the acyl species of PtdIns(4,5) P_2 present in purified splenic CD4⁺ T-cells isolated from wild-type and *Fat-1* mice. Total PtdIns(4,5) P_2 in purified *Fat-1* splenic CD4⁺ T-cells was decreased by 50% compared with wild-type ($P=0.04$, Figure 1A). In addition, there were significant decreases in the C_{18:0,20:4} species ($P0.02$) and an increase in the C_{18:1,20:4} species of PtdIns(4,5) P_2 ($P0.04$) in *Fat-1* splenic CD4⁺ T-cells (Figure 1B). Using two additional methods [PtdIns(4,5) P_2 mass kit, and indirect anti-PtdIns(4,5) P_2 ELISA], we further validated our observations, demonstrating a decrease in the total PtdIns(4,5) P_2 content in *Fat-1* splenic CD4⁺ T-cells (Supplementary Figure S2 at <http://www.BiochemJ.org/bj/443/bj4430027add.htm>).

Examination of PtdIns P molecular species revealed a decrease only in the C_{16:0,20:4} species ($P=0.03$, Supplementary Figure S1). There were no effects on the C_{18:0,20:4} ($P=0.09$) or C_{18:1,20:4} PtdIns P ($P=0.53$), or the minor species of PtdIns P ($P=0.32$) between wild-type and *Fat-1* CD4⁺ T-cells (Supplementary Figure S2). Furthermore, the total PtdIns P level was not significantly different between genotypes ($P=0.17$). Taken together, these results demonstrate that the basal PtdIns(4,5) P_2 concentration is decreased in purified splenic CD4⁺ T-cells isolated from *Fat-1* mice.

Metabolism of PtdIns(4,5) P_2 upon T-cell activation is altered in *Fat-1* CD4⁺ T-cells

We have demonstrated previously that *Fat-1* CD4⁺ T-cells have a suppressed activation status, e.g. decreased localization and phosphorylation of PLC β 1 at the IS [32]. Therefore we examined the metabolism of PtdIns(4,5) P_2 upon T-cell activation by anti-CD3/anti-CD28 or PMA/ionomycin in purified splenic *Fat-1* CD4⁺ T-cells. In both stimulation conditions, *Fat-1* CD4⁺ T-cells failed to respond to stimulation (Figure 2). In contrast, in wild-type CD4⁺ T-cells activated with anti-CD3/anti-CD28, the level of PtdIns(4,5) P_2 decreased by 50% in 5 min ($P=0.05$), followed by a recovery phase of ~30 min. *Fat-1* CD4⁺ T-cells showed no change upon activation with anti-CD3/anti-CD28. Similarly, PtdIns(4,5) P_2 levels in wild-type CD4⁺ T-cells activated with PMA/ionomycin were decreased by 50% within 5 min of stimulation ($P=0.008$). However, PtdIns(4,5) P_2 levels did not recover possibly due to the non-physiological (TCR-independent) activation of the T-cells. In contrast, *Fat-1* CD4⁺ T-cells showed no response to PMA/ionomycin.

Actin remodelling following anti-CD3/anti-CD28 stimulation is suppressed in *Fat-1* CD4⁺ T-cells

Since we observed both a decrease in the basal concentration of PtdIns(4,5) P_2 and a suppression in PtdIns(4,5) P_2 metabolism, we subsequently determined whether PtdIns(4,5) P_2 -mediated actin remodelling was altered in splenic *Fat-1* CD4⁺ T-cells. For this purpose, purified splenic CD4⁺ T-cells were isolated from wild-type and *Fat-1* mice, stimulated with anti-CD3/anti-CD28 for 30 min, and actin morphology was examined using Alexa Fluor® 568-conjugated phalloidin immunofluorescence (Figure 3A). When compared with unstimulated wild-type CD4⁺ T-cells, there was a 2.5-fold increase ($P<0.05$) in actin fluorescence intensity in anti-CD3/anti-CD28-stimulated wild-type CD4⁺ T-cells (Figure 3B). In contrast, there was no difference ($P>0.05$) between stimulated and unstimulated CD4⁺ T-cells isolated from *Fat-1* mice. These data indicate that actin remodelling is defective in activated splenic CD4⁺ T-cells isolated from *Fat-1* mice.

WASP recruitment to the IS is suppressed in *Fat-1* CD4⁺ T-cells

WASP, a well-characterized actin-regulatory protein, is known to be localized at the IS upon stimulation [38] and to be regulated at the plasma membrane by PtdIns(4,5) P_2 [39]. Since PtdIns(4,5) P_2 metabolism and actin remodelling were both defective in *Fat-1* CD4⁺ T-cells, we examined whether WASP recruitment to the IS was also decreased in stimulated *Fat-1* CD4⁺ T-cells. Actin recruitment at the IS, as measured by fluorescence intensity per μm^2 , was decreased in *Fat-1* CD4⁺ T-cells (Figure 4A), confirming our previous results ([32] and Figure 3). Under unstimulated conditions, WASP levels were not different between wild-type and *Fat-1* CD4⁺ T-cells, as assessed by immunoblot analysis (Supplementary Figure S3C at <http://www.BiochemJ.org/bj/443/bj4430027add.htm>); similarly, whole-cell levels of WASP were not different between genotypes upon anti-CD3/anti-CD28-coated bead stimulation (Supplementary Figure S3B). However, upon measuring the fluorescence intensity at the IS, there was a 2.5-fold decrease in the fluorescence intensity per μm^2 , suggesting that the recruitment of WASP to the IS in *Fat-1* CD4⁺ T-cells was impaired (Figure 4B). Co-localization between Alexa Fluor® 568 [F-actin (filamentous actin)] and Alexa Fluor® 647 (WASP) at the IS, as determined by Pearson's correlation coefficient, showed a decrease in *Fat-1* CD4⁺ T-cells [wild-type = 0.45 ± 0.28 ($n = 33$)]. *Fat-1* = 0.31 ± 0.22 ($n = 35$), Supplementary Figure S3D]. To further corroborate the impairment of WASP recruitment to the IS in *Fat-1* CD4⁺ T-cells, we isolated IS fractions using immunoisolation [37] to biochemically probe the level of WASP at the IS upon anti-CD3/anti-CD28 stimulation. Examination of the IS fraction revealed that, in wild-type CD4⁺ T-cells, the enrichment factor between the IS compared with input was 1.7 ± 0.5 ($n = 3$), whereas the enrichment factor in *Fat-1* CD4⁺ T-cells was 0.4 ± 0.1 ($n = 4$) (Figure 4D). These results demonstrate that the suppressed actin remodelling in *Fat-1* CD4⁺ T-cells is correlated with decreased WASP recruitment to the IS upon T-cell activation.

Defects in actin remodelling following anti-CD3/anti-CD28 stimulation are rescued by incubation with exogenous PtdIns(4,5) P_2 in *Fat-1* CD4⁺ T-cells

Since *Fat-1* CD4⁺ T-cells exhibited decreased basal PtdIns(4,5) P_2 (Figure 1), we determined whether pre-incubation of *Fat-1* CD4⁺ T-cells with exogenous PtdIns(4,5) P_2 would 'rescue' the defects in actin remodelling observed in *Fat-1* CD4⁺ T-cells (Figure 3). Treatment of *Fat-1* CD4⁺ T-cells with exogenous PtdIns(4,5) P_2 at increasing concentrations showed a dose-dependent rescue of actin remodelling following stimulation with anti-CD3/anti-CD28 (Figure 5). Significantly, incubation of *Fat-1* CD4⁺ T-cells with 1.25 μM and 2.5 μM exogenous PtdIns(4,5) P_2 restored actin remodelling to the level seen in wild-type CD4⁺ T-cells (Figure 5B). These results not only demonstrate the direct role of PtdIns(4,5) P_2 in regulating actin remodelling upon CD4⁺ T-cell activation, but also show that defects in *Fat-1* CD4⁺ T-cell actin remodelling can be rescued using exogenous PtdIns(4,5) P_2 .

CD4⁺ T-cells isolated from mice fed a DHA triacylglycerol-enriched diet exhibit a phenotype similar to *Fat-1* CD4⁺ T-cells

The effect of exogenous (dietary) DHA on PtdIns(4,5) P_2 -dependent actin remodelling was also examined. For this purpose, mice were fed on a 4% DHA triacylglycerol-enriched diet in an attempt to mimic the phenotype observed in *Fat-1* CD4⁺ T-cells. No difference was observed between the body masses of animals fed on a 5% corn oil diet (control, contains no $n - 3$ PUFA) or the 4% DHA triacylglycerol-enriched diet ($P > 0.05$, Supplementary Figure S4 at Figure 6A, $P = 0.04$) compared with control. Furthermore, there was a suppression of PtdIns(4,5) P_2 metabolism following anti-CD3/anti-CD28 stimulation and PMA/ionomycin stimulation in purified splenic CD4⁺ T-cells from mice fed on the 4% DHA (Figures 6B and 6C, $P = 0.005$ and 0.01 respectively) compared with the control diet, as revealed by two-way ANOVA. Interestingly, actin morphology remained unchanged in splenic CD4⁺ T-cells isolated from animals fed on a DHA-enriched diet, whereas splenic CD4⁺ T-cells isolated

from animals fed on the control diet showed a significant increase upon anti-CD3/anti-CD28 stimulation ($P < 0.05$, Figure 7). These results indicate that CD4⁺ T-cells isolated from *Fat-1* mice or animals fed on a 4% DHA triacylglycerol-enriched diet exhibit similar phenotypes with regard to PtdIns(4,5) P_2 metabolism and actin remodelling.

DISCUSSION

In the present study, we have shown that $n-3$ PUFA, i.e. DHA, critically regulate PtdIns(4,5) P_2 -dependent actin remodelling in CD4⁺ T-cells. This novel effect was associated with decreased levels of PtdIns(4,5) P_2 in unstimulated CD4⁺ T-cells and suppressed metabolism of PtdIns(4,5) P_2 upon anti-CD3/anti-CD28 or PMA/ionomycin stimulation.

PtdIns(4,5) P_2 can be synthesized via two pathways: (i) the *de novo* pathway (Kennedy pathway) and (ii) the remodelling pathway (Lands' cycle). Since the incorporation of PUFA at the *sn*-2 position of PtdIns(4,5) P_2 is predominantly driven by the Lands' cycle rather than the Kennedy cycle [40,41], the phospholipid composition of the membrane can potentially influence the acyl composition of phosphoinositides [42]. Indeed, it has been shown that platelet 1-acyl-glycero-3-phosphoinositol acyltransferase, which catalyses the condensation between fatty acyl CoA and lysophospholipid, prefers $n-6$ PUFA over $n-3$ PUFA [43]. The results of the present study support this model, as the presence of $n-3$ PUFA decreased the C_{16:0,20:4} and C_{18:0,20:4} species of PtdIns(4,5) P_2 , while concomitantly increasing the C_{18:1,20:4} species of PtdIns(4,5) P_2 (Figure 1).

With regard to PtdIns(4,5) P_2 metabolic turnover plots (Figure 2), wild-type data are similar to previously published work [44]. Furthermore, PtdIns(4,5) P_2 metabolism in *Fat-1* and 4% DHA CD4⁺ T-cells are consistent with published results showing that porcine cardiac myocytes incubated with DHA exhibited no change in Ins(1,4,5) P_3 generation after stimulation by an α_1 -adrenoreceptor agonist [45]. Similarly, we have shown previously that diacylglycerol production in purified murine T-lymphocytes isolated from mice fed on dietary EPA and DHA is suppressed upon stimulation [46]. The PtdIns(4,5) P_2 metabolic profile in *Fat-1* CD4⁺ T-cells also corroborates our previous data that PLC β_1 and phosphorylated PLC β_1 are down-regulated in T-cells at the IS [32].

The magnitude and kinetics of PtdIns(4,5) P_2 metabolism are important for actin remodelling, as demonstrated in various cell types and physiological processes [24-26]. For example, the overexpression of PIP5K (PtdIns4 P 5-kinase), which increases the availability of PtdIns(4,5) P_2 , perturbs stress actin fibres and membrane ruffling, indicative of defects in actin reorganization [47]. Our data reveal that the distortion of PtdIns(4,5) P_2 levels is associated with the suppression of actin remodelling in *Fat-1* and 4% DHA-enriched CD4⁺ T-cells (Figures 3 and 7). We also demonstrate that the defects in actin remodelling observed in *Fat-1* CD4⁺ T-cells can be rescued by the introduction of exogenous PtdIns(4,5) P_2 (Figure 5). It is interesting to note that mitochondrial translocation in CD4⁺ T-cells is inhibited by latrunculin, a drug that sequesters actin monomers, preventing actin polymerization [48]. We have shown previously that mitochondrial translocation after stimulation in *Fat-1* CD4⁺ T-cells is suppressed [30], thus our results that actin remodelling is suppressed in *Fat-1* CD4⁺ T-cells may partially explain the lack of mitochondrial translocation upon CD4⁺ T-cell stimulation.

WASP, an actin-regulatory protein, is activated by PtdIns(4,5) P_2 through interaction between the basic domain of WASP and the acidic PtdIns(4,5) P_2 to release WASP from its autoinhibitory conformation [19,39]. Interestingly, activation of N-WASP (neuronal WASP), a member of the WASP family, is dependent upon PtdIns(4,5) P_2 density, i.e.

increased PtdIns(4,5) P_2 density leads to hyperactivation of N-WASP *in vitro* [49]. Furthermore, depletion of the PtdIns(4,5) P_2 fraction in the raft pool resulted in decreased T-cell capping upon T-cell activation [27]. One can speculate, therefore, that PtdIns(4,5) P_2 metabolism upon activation of CD4⁺ T-cell leads to a local increase in PtdIns(4,5) P_2 density, which results in the recruitment and activation of WASP at the IS and subsequent actin remodelling. In the presence of *n* – 3 PUFA, not only is basal PtdIns(4,5) P_2 decreased, but PtdIns(4,5) P_2 fails to metabolize upon activation, leading to suppressed WASP recruitment to the IS and actin remodelling (Figure 4). Further experiments are required to test this hypothesis.

In the 4% DHA triacylglycerol-enriched diet, DHA contributed approximately 5% toward the total energy (kcal) intake. In a typical Greenland Inuit diet, *n* 3 PUFA constitutes approximately 2.7-6.3% of daily energy-[50-52]. Thus our 4% DHA diet is within the physiological range achievable through diet alone. In humans, comparable intakes could also be achieved through the ingestion of DHA supplements. At this level, we did not observe any adverse effects, as food intake and changes in body mass were similar between the dietary groups (results not shown, and Supplementary Figure S4).

In summary, the results of the present study demonstrate the novel effects of *n* – 3 PUFA on critical mechanisms of early T-cell activation (Figure 8). We have demonstrated previously that *n* – 3 PUFA can suppress T-cell activation, in part, by affecting recruitment and activation of signalling proteins such as PLC 1, PKC (protein kinase C) and F-actin, as well as impairing mitochondrial translocation which is necessary to sustain Ca²⁺ signalling for nuclear NF- B and AP-1 (activator protein 1) activation, and IL (interleukin)-2 secretion [30-32]. We extend this model by demonstrating that *n* – 3 PUFA, such as DHA, can also affect PtdIns(4,5) P_2 -dependent actin remodelling by decreasing steady-state PtdIns(4,5) P_2 levels, suppressing PtdIns(4,5) P_2 metabolism upon stimulation and inhibiting PtdIns(4,5) P_2 -dependent actin remodelling. In addition, a mechanism by which *n* – 3 PUFA suppress PtdIns(4,5) P_2 -dependent actin remodelling is through decreased WASP recruitment to the IS. In contrast with commonly used pharmacological perturbations, our *in vivo* genetic and dietary intervention studies carry significant biological relevance. Overall, our findings highlight a novel modality by which *n* – 3 PUFA influence membrane micro-organization, thereby modulating biological responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

DHA docosahexaenoic acid

EPA	eicosapentaenoic acid
ERM	e-zrin, radixin and moesin
F-actin	filamentous actin
GADS	Grb2 (growth-factor-receptor-bound protein 2)-related adaptor protein
IS	immunological synapse
LAT	linker for activation of T-cells
Lo	liquid-ordered
NF- B	nuclear factor B
PAK	p21-activated kinase
PKC	protein kinase C
PLC 1	phospholipase C 1
PUFA	polyunsaturated fatty acid(s)
ROI	region of interest
SLP76	SH2 (Src homology 2) domain-containing leucocyte protein of 76 kDa
TCR	T-cell receptor
WASP	Wiskott-Aldrich syndrome protein
N-WASP	neuronal WASP
ZAP-70	-chain (TCR)-associated protein kinase of 70 kDa

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Figure 1. Total PtdIns(4,5) P_2 is decreased in *Fat-1* CD4⁺ T-cells

(A) Relative PtdIns(4,5) P_2 was decreased by 50% in *Fat-1* CD4⁺ T-cells. CD4⁺ T-cells were isolated from wild-type and *Fat-1* spleens ($n = 8$ for *Fat-1*; $n = 12$ for wild-type). CD4⁺ T-cell populations from two spleens were pooled for analysis using MS as described in the Experimental section. Total PtdIns(4,5) P_2 was quantified following comparison with an internal standard and normalized relative to total CD4⁺ T-cells counted using a Coulter Counter. Relative PtdIns(4,5) P_2 levels were compared by normalizing to wild-type PtdIns(4,5) P_2 . (B) PtdIns(4,5) P_2 molecular species were altered in *Fat-1* CD4⁺ T-cells. PIP₂, PtdIns(4,5) P_2 .

A CD3/CD28 **B PMA/Ionomycin**

Figure 2. Metabolism of PtdIns(4,5) P_2 upon (A) anti-CD3/anti-CD28 and (B) PMA/ionomycin stimulation was suppressed in *Fat-1* $CD4^+$ T-cells

Splenic $CD4^+$ T-cells were isolated from wild-type and *Fat-1* mice ($n = 4$). $CD4^+$ T-cells (5×10^5) were seeded in 96-well plates containing plated anti-CD3 and anti-CD28 for the indicated times. PtdIns(4,5) P_2 was subsequently extracted using acidic organic solvents, dried under nitrogen and dissolved in $1 \times$ PBS containing 0.0025% protein stabilizer. PtdIns(4,5) P_2 was detected using indirect anti-PtdIns(4,5) P_2 ELISA as described in the Experimental section. Absolute values were determined using a standard curve and normalized relative to wild-type PtdIns(4,5) P_2 . A two-tailed Student's *t* test was used to compare individual times points between genotypes (* $P < 0.05$ between genotypes at specific time points).

Figure 3. Actin remodelling upon anti-CD3/anti-CD28 stimulation is suppressed in *Fat-1* CD4⁺ T-cells

(A) Representative Alexa Fluor® 568-conjugated phalloidin immunofluorescence images of wild-type and *Fat-1* CD4⁺ T-cells seeded on to slides containing only poly-L-lysine (unstimulated) or on slides containing anti-CD3/anti-CD28 (stimulated) for 30 min. Images were pseudo-coloured with increasing intensity from blue to red. (B) Quantification of actin remodelling as assessed by Alexa Fluor® 568-conjugated phalloidin fluorescence. Purified splenic CD4⁺ T-cells ($n = 4$ per genotype) were seeded, fixed, permeabilized and labelled as described in the Experimental section. ROIs were drawn around individual cells, and background was subtracted by drawing ROIs not occupied by cells. Fluorescence values were divided by the number of cells present in each field. Six fields were obtained per group, with each field containing between three and seven cells. Four independent experiments were conducted. Different letters denote a significant difference at $P < 0.05$. A.U., arbitrary unit; DIC, differential interference contrast.

Figure 4. WASP localization to the IS and co-localization with actin at the IS are decreased in *Fat-1* CD4⁺ T-cells

(A) Actin remodelling at the IS was suppressed in *Fat-1* CD4⁺ T-cells. Purified splenic CD4⁺ T-cells (n 3 per genotype) were seeded, stimulated with anti-CD3/anti-CD28-coated beads, fixed, permeabilized and labelled as described in the Experimental section. ROIs were drawn and fluorescence=values divided by the area of the IS. In total, 10-15 cells were analysed per genotype. Three independent experiments were conducted. A two-tailed Student's t test was used to compare between genotypes. (B) WASP recruitment to the IS was suppressed in *Fat-1* CD4⁺ T-cells. Experimental details are given above. (C) Representative immunofluorescence images of purified splenic CD4⁺ T-cells isolated from wild-type and *Fat-1* mice. Cells were seeded on to poly-L-lysine and stimulated with anti-CD3/anti-CD28-coated beads. The ROI was drawn as an oval or polygon (white broken line) as shown in the wild-type panels. (D) WASP recruitment to the IS as assessed by immunoprecipitation. IS fractions from wild-type (WT) (n = 3) and *Fat-1* (n = 4) mice were prepared as described in the Experimental section. WASP (66 kDa) was probed in both the IS and input fractions. The enrichment factor was determined by calculating the ratio of the band intensities from the IS divided by the input. A two-tailed Student's t test was used to compare between genotypes. AU, arbitrary unit; DIC, differential interference contrast.

Figure 5. Incubation of *Fat-1* CD4⁺ T-cells with exogenous PtdIns(4,5)P₂ can rescue defects in actin remodelling following anti-CD3/anti-CD28 stimulation

Fat-1 CD4⁺ T-cells were isolated and incubated with PtdIns(4,5)P₂ at various concentrations (0.5, 1.25 and 2.5 μM) or PBS (0 μM) for 1 h prior to stimulation with plated anti-CD3/anti-CD28 for 30 min. Cells were analysed as described in Figure 3. Images were pseudo-coloured with increasing intensity from blue to red. A two-tailed Student's *t* test was used to compare stimulation within specific concentrations [**P* < 0.05 at specific concentrations of PtdIns(4,5)P₂]. A.U., arbitrary unit; DIC, differential interference contrast; PIP₂, PtdIns(4,5)P₂.

Figure 6. CD4⁺ T-cells isolated from mice fed on a 4% DHA triacylglycerol-enriched diet exhibit altered PtdIns(4,5)P₂ metabolism and actin remodelling upon anti-CD3/anti-CD28 stimulation (A) Relative PtdIns(4,5)P₂ levels in unstimulated splenic CD4⁺ T-cells isolated from mice fed on a 5% corn oil diet (control, *n* = 7) or 4% DHA triacylglycerol-enriched (*n* = 8) diet were determined by indirect anti-PtdIns(4,5)P₂ ELISA as described in the Experimental section. Relative PtdIns(4,5)P₂ levels were compared by normalizing to wild-type PtdIns(4,5)P₂. (B) PtdIns(4,5)P₂ levels upon anti-CD3/anti-CD28 stimulation in CD4⁺ T-cells isolated from mice fed on a 5% corn oil or a 4% DHA triacylglycerol-enriched diet. CD4⁺ T-cells were seeded in 96-well plates with plate-bound anti-CD3 and anti-CD28 as described in the Experimental section. A two-way ANOVA indicated statistical significance between the two diet groups (*P* = 0.005). (C) PtdIns(4,5)P₂ levels upon PMA/ionomycin stimulation in CD4⁺ T-cells isolated from mice fed on a 5% corn oil or 4% DHA triacylglycerol-enriched diet. A two-way ANOVA indicated statistical significance between the diet groups (*P* 0.01). A two-tailed Student's *t* test was used to compare individual times points between=treatment groups (**P* < 0.05 at specific time points). CO, corn oil; PIP₂, PtdIns(4,5)P₂.

Figure 7. Actin remodelling upon anti-CD3/anti-CD28 stimulation is suppressed in CD4⁺ T-cells isolated from animals fed on a 4% DHA triacylglycerol-enriched diet

(A) Representative immunofluorescence images of purified splenic CD4⁺ T-cells isolated from (control) 5% corn oil (CO)- and 4% DHA-fed mice. Cells were unstimulated or incubated on slides with anti-CD3/anti-CD28 (stimulated) for 30 min. Images were pseudo-coloured with increasing intensity from blue to red. (B) Quantification of actin remodelling. Refer to Figure 3(B) for experimental details. Different letters denote $P < 0.05$. A.U., arbitrary units; DIC, differential interference contrast.

Figure 8. *n* – 3 PUFA alter early steps of T-cell activation

In wild-type CD4⁺ T-cells, upon stimulation with anti-CD3 (and co-stimulatory signal from CD28), the TCR signosome composed of Lck, ZAP-70, LAT, GADS, SLP76, NCK, ITK, VAV1, PAK and PLC-γ1 is formed, recruiting additional proteins such as PLC-γ1. PLC-γ1 hydrolyses the lipid mediator PtdIns(4,5)P₂ to diacylglycerol (DAG) and Ins(1,4,5)P₃, leading to induction of Ca²⁺ signalling and the PKC signalling pathway. The hydrolysis of PtdIns(4,5)P₂ is also important for actin remodelling by releasing actin-regulating proteins localized at the plasma membrane. Additionally, PtdIns(4,5)P₂ is phosphorylated by class I PI3K (phosphoinositide 3-kinase) to generate PtdIns(3,4,5)P₃ which also triggers actin remodelling. WASP is recruited to the plasma membrane and is activated by PtdIns(4,5)P₂ to regulate actin remodelling. These pathways synergistically regulate actin remodelling at the plasma membrane for mitochondrial translocation and T-cell activation. We have shown previously that *n* – 3 PUFA suppress total and phosphorylated PLC-γ1 translocation to the IS (dotted hammerheads signify suppression) [32] and mitochondrial translocation to the IS [30]. We now show that *n* – 3 PUFA, such as DHA, can suppress (i) the basal level of PtdIns(4,5)P₂ in unstimulated CD4⁺ T-cells, (ii) the metabolism of PtdIns(4,5)P₂ upon anti-CD3/anti-CD28 or PMA/ionomycin stimulation, (iii) WASP recruitment to the IS and (iv) actin remodelling upon anti-CD3/anti-CD28 stimulation. The suppression of these early immediate events in T-cell activation leads to inhibition of T-cell function, and thus may suppress inflammatory responses.