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The Docosatriene Protectin D1 Is Produced by T_H2 Skewing and Promotes Human T Cell Apoptosis via Lipid Raft Clustering*

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Docosahexaenoic acid, a major ω -3 fatty acid in human brain, synapses, retina, and other neural tissues, displays beneficial actions in neuronal development, cancer, and inflammatory diseases by mechanisms that remain to be elucidated. In this study we found, using lipid mediator informatics employing liquid chromatography-tandem mass spectrometry, that (10,17S)-docosatriene/neuroprotectin D1, now termed protectin D1 (PD1), is generated from docosahexaenoic acid by T helper type 2-skewed peripheral blood mononuclear cells in a lipoxygenase-dependent manner. PD1 blocked T cell migration *in vivo*, inhibited tumor necrosis factor α and interferon- γ secretion, and promoted apoptosis mediated by raft clustering. These results demonstrated novel anti-inflammatory roles for PD1 in regulating events associated with inflammation and resolution.

Chemical mediators and autacoids, such as the local-acting lipid mediators derived from arachidonic acid, are well appreciated regulators of host defense, coagulation, inflammation, and cancer (1, 2). The family of bioactive eicosanoids include leukotriene B₄, for example, a major mediator of acute inflammation produced by neutrophils recently found to also regulate the recruitment of cytotoxic effector T cells to inflamed tissue (3). These findings expand the role and actions of eicosanoids from innate to acquired immunity. Over the past 25 years, numerous studies have reported that ω -3 polyunsaturated fatty acids, administered as a dietary supplement, have therapeutic impact on human diseases such as atherosclerosis, arthritis, and asthma (4–8). DHA,⁴ a major ω -3 polyunsaturated fatty acid, is particularly prevalent

in neuronal tissues, and its content in maternal breast milk correlates with both neural development end points and immune status in the fetus (9, 10). DHA is also essential for proper T cell development in neonates (11) and regulates T cell functions, with a polarizing preference toward the T_H2 phenotype as well as promotion of apoptosis in T_H1-polarized CD4⁺ T cells (12, 13). Consequently, T_H2 cells are potent suppressors of inflammatory and autoimmune disorders, particularly in the central nervous system (14). However, the molecular mechanisms of the diverse actions of DHA in each of these vital systems still remain to be elucidated.

Along these lines, the question of whether DHA is a precursor to novel potent lipid mediators was of interest. This laboratory recently identified several novel 17-hydroxyl-containing bioactive chemical mediators derived from DHA that were termed docosatrienes and 17S series resolvins (15, 16). Neuroprotectin D1 ((10,17S)-dihydroxydocosatriene; NPD1) appears to be a major bioactive effector from this novel series of docosatrienes that possesses a conjugated triene system with alcohol groups at the 10 and 17 carbon positions as distinguishing features (17). At picogram to nanogram levels, NPD1 reduces the infiltration of polymorphonuclear cells (PMN) into both peritoneal exudates and murine ischemic brain (*i.e.* experimental stroke), resulting in both anti-inflammation and neuroprotection (15, 16). NPD1 was also found recently to inhibit oxidative stress-induced apoptosis of human retinal pigment epithelial cells (18).

Considerable evidence indicates that both the generation and resolution of a localized inflammatory or immune response are governed by the migration of leukocytes into the site of injury and proliferation *in situ* and then by the removal of these cells after antigenic clearance (19). Aberrant leukocyte behavior can lead to chronic inflammation (20–22). One of the important mechanisms essential to resolution of the immune response involves the clearance of T cells from the local microenvironment. Hence the efflux or death of T cells *in situ* significantly contributes to the resolution of inflammatory responses. Conversely, decreased clearance with accumulation of T cells and/or additional inflammatory cells may result in chronic inflammation (23, 24). Although the mechanisms mediating T cell clearance and resolution are still not fully appreciated, recent results indicate that cytokine deprivation and/or their decreased production by T cells plays an essential role in the clearance of T cells from inflamed tissues and consequently in the resolution of inflammation (25, 26).

Here we report that human T_H2-skewed PBMC specifically express 15-LO type 1 that converts DHA to novel docosatrienes by serving as a

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⁴ The abbreviations used are: DHA, C22:6, docosahexaenoic acid; ATLa, aspirin-triggered lipoxin A₄ analog; CTX, cholera toxin; (10,17S)-DT, (10,17S)-docosatriene ((10,17S)-dihydroxydocosahexaenoic acid); (17S)-HDHA, (17S)-hydroxydocosahexaenoic acid; (18R)-THEPE, (18R)-trihydroxyeicosapentaenoic acid; IFN, interferon; IL, interleukin; LC-MS-MS, liquid chromatography-tandem mass spectrometry; LO, lipoxygenase; PBMC, peripheral blood mononuclear cells; PMN, polymorphonuclear leukocytes; PD1, protectin D1; NPD1, neuroprotectin D1; TUNEL, terminal deoxynucleotidyltransferase-mediated

dUTP nick-labeling; T_H, T helper type; TNF, tumor necrosis factor; FITC, fluorescein isothiocyanate; PE, phycoerythrin; siRNA, small interfering RNA; PBS, phosphate-buffered saline.

DHA Pathways and Actions of Protectin D1 with Human PBMC

17-lipoxygenase with DHA as substrate. Neuroprotectin D1 is a major product of this novel pathway that regulates leukocyte migration and protein deposition in inflamed tissues, as well as TNF α and IFN γ secretion by activated T cells. This DHA-derived product is also produced by neural and inflammatory tissues (15–17) and is termed neuroprotectin D1 (18). Because this docosatriene possesses a broader scope of formation and actions and is not restricted to neuronal tissue, we proposed protectin D1 (PD1) in recognition of its wider range of activities and functions in biological systems other than neural systems. Also, PD1 promotes T cell apoptosis via the formation of lipid raft-encoded signaling complexes.

EXPERIMENTAL PROCEDURES

Reagents—The following materials were obtained as indicated: DHA, bovine serum albumin, Histopaque-1077, nystatin, FITC-labeled CTX, and alkaline phosphatase substrate from Sigma; ^{14}C -labeled DHA from ARC (St. Louis, MO); RPMI 1640, fetal bovine serum, glutamine, penicillin, and streptomycin from BioWhittaker (Walkersville, MD); purified and biotinylated mouse anti-human TNF α and IFN γ , and FITC- or PE-conjugated mouse IgG, and GolgyPlugTM from Pharmingen; FITC-conjugated mouse anti-IFN γ and PE-conjugated mouse anti-IL-4 from eBioscience (San Diego, CA); purified Texas Red-conjugated rabbit anti-mouse IgG and streptavidin-conjugated alkaline phosphatase from Jackson ImmunoResearch (West Grove, PA); Fas ligand from Upstate Biotechnology, Inc. (Lake Placid, NY); and purified mouse anti-Fas from Oncogen (Cambridge, MA). ATLa and (18R)-THEPE were prepared by total organic synthesis and characterized, including by magnetic resonance spectroscopy, as indicated in Refs. 27 and 4, respectively. PD1 was prepared by biologic synthesis and purified as described previously (15).

Preparation of Human PBMC and T Cells—PBMC were freshly isolated from the venous blood of healthy volunteers by Histopaque-1077 gradient centrifugation as in Ariel *et al.* (28). T cells were isolated by incubating PBMC for 2 h on a plastic surface to deplete the monocytes. The nonadherent cells were collected and separated on a human CD3⁺ enrichment column (R & D Systems, Minneapolis, MN) according to the manufacturer's instructions and were cultured for use. The purity of these T cells was typically >92%.

Human PBMC were skewed to a T_H1 or T_H2 phenotype by culturing with concanavalin A (1 $\mu\text{g}/\text{ml}$), IL-12 (5 ng/ml), IFN γ (50 ng/ml), and anti-IL-4 (1 $\mu\text{g}/\text{ml}$) or with concanavalin A (1 $\mu\text{g}/\text{ml}$), IL-4 (25 ng/ml), and anti-IFN γ (1 $\mu\text{g}/\text{ml}$), respectively. On days 3 and 6 of culture, IL-2 (5 ng/ml) was added to both cultures, and T_H2 cells were added with anti-IFN γ (1 $\mu\text{g}/\text{ml}$). To confirm the skewing, PBMC were collected on day 6 and activated by immobilized anti-CD3 (5 $\mu\text{g}/\text{ml}$) + anti-CD28 (2 $\mu\text{g}/\text{ml}$) in the presence of GolgyPlugTM (1:500). After 5 h the cells were fixed, permeabilized, and stained by FITC-conjugated mouse anti-IFN γ and PE-conjugated mouse anti-IL-4 and analyzed by fluorescence-activated cell sorter. FITC- or PE-conjugated mouse IgG were used as controls. The stained cells were used for analysis by FACSsort (BD Biosciences).

Expression and Function of 15-LO in Skewed PBMC—Skewed human PBMC (toward T_H1 or T_H2 phenotype) were collected after 3–9 days of culture, and their RNA was extracted with Trizol reagent (Invitrogen). RNA was reverse-transcribed for 30 min (50 °C) using the ThermoScript system (Invitrogen), which was followed by 40 cycles of PCR with specific primers for human glyceraldehyde-3-phosphate dehydrogenase, 15-LO, and 5-LO using the HotStartTaq-MasterMix kit (Qiagen, Chatsworth, CA). Amplified cDNA fragments were separated by agarose gel electrophoresis, and bands were visualized by ethidium bromide staining. The appropriate nucleotide primers were used to amplify and

sequence human 15-LO type 1 (sense, 5'-CAGCCTAGGCAACGTG-GTGAAACC-3'; antisense, 5'-CCTCCTGGGTCTCTCTGTC-CTCA-3') and 5-LO (sense, 5'-ATCAGGACGGACATGAGGAA-CAGG-3'; antisense, 5'-CCAGGAACAGCTCGTTTTCTG-3'). Sequencing was done by the Brigham and Women's Hospital Automatic Genotyping and Sequencing Facility. Expression of 15-LO-1 protein was determined by lysing cells and subjecting the lysate to SDS-PAGE followed by transfer to nitrocellulose membrane and immunoblotting with anti-15-LO-1 (Cayman Chemical, Ann Arbor, MI).

LC-MS-MS Analysis—Skewed human PBMC (T_H1- or T_H2-directed) were collected on days 3–4 and incubated with or without DHA or IL-4 (20 μM or 10 ng/ml respectively, 60 min, 37 °C). In other experiments, PBMC were transfected with 36 pmol of a mixture of three siRNA species directed against 15-LO-1 (ID numbers 2306, 2399, and 2486; Ambion, Austin, TX) or control siRNA (nonspecific control IX siRNA; Dharmacon Research, Lafayette, CO) for 2 h, and then were cultured under T_H2-skewing conditions for 4 days with daily additions of the same siRNA species. On day 3 the cells were added with IL-4 and anti-IFN γ as in the initial culturing. Next, the cells were harvested, and samples of the cells were taken for determination of 15-LO-1 expression using FACSsort analysis with sheep anti-15-LO-1 and PE-conjugated goat anti-sheep antibodies. Sheep IgG was used as a negative control. The remaining cells were incubated with DHA as above. The incubation was stopped and extracted with high performance liquid chromatography-grade MeOH. The products were isolated with C18 solid-phase extraction cartridges (Alltech Associates, Deerfield, IL) for identification and then subjected to LC-MS-MS as described in Hong *et al.* (15). In brief, an MS-MS-based informatic analysis of lipid mediators was performed to determine the production of PD1, (17S)-HDHA, and other DHA-derived oxygenated products using a Finnigan LCQ liquid chromatography ion trap tandem mass spectrometer (San Jose, CA) equipped with a LUNA C18-2 (100 \times 2 mm \times 5 μm) column, and a photodiode array detector that monitored UV absorbance before the samples entered the MS-MS. The percentage of inhibition of product formation was calculated as follows: (1 – (amount of product with control siRNA/amount of product with 15-LO-1 siRNA)) \times 100.

Murine Peritonitis—Peritonitis was performed using 6–8-week-old FVB male mice (Charles River Breeding Laboratories, Wilmington, MA) fed with Laboratory Rodent Diet 5001 (Purina Mills), as described previously (17). Briefly, mice were first anesthetized with isoflurane and injected intravenously with 100 ng of PD1, DHA, or vehicle and then injected with 1 ml of zymosan A (1 mg/ml) into the peritoneum. After 2 h the mice were killed, and the peritoneal lavages were rapidly collected for enumeration of leukocytes. For identification of T and B cells in the lavage, the recovered cells were double stained with FITC-conjugated anti-mouse CD3e and PE-conjugated anti-mouse CD19 (0.5 and 0.2 $\mu\text{g}/0.5 \times 10^6$ cells, clones 145-2C11 and 1D3, respectively, Pharmingen) and analyzed by FACSsort.

In some mice, the peritoneal tissues were removed and fixed in 10% neutral formalin for morphologic examination. The peritoneal lining was then excised, and histological sections were stained with hematoxylin and eosin.

T Cell Incubations—Cytokines (TNF α and IFN γ) produced by human T cells were determined as described in Ariel *et al.* (28). Briefly, human peripheral blood T cells were incubated with increasing concentrations of PD1 or vehicle (0.05% EtOH) for 6 h at 37 °C and then in 48-well plates coated with anti-CD3 + anti-CD28 (2 $\mu\text{g}/\text{ml}$ each) or anti-CD3 alone and cultured for 41 h. The supernatants were collected from the wells and evaluated for TNF α and IFN γ content by a standard

enzyme-linked immunosorbent assay. The percentage inhibition for CD28 co-stimulation was calculated as follows: (amount of cytokine induced by (anti-CD3 + anti-CD28) – amount of cytokine induced by (anti-CD3 + anti-CD28 + PD1))/(amount of cytokine induced by (anti-CD3 + anti-CD28) – amount of cytokine induced by anti-CD3).

T Cell Apoptosis—Apoptosis of human peripheral blood T cells was detected using the *In Situ* Cell Death Detection Kit (Roche Applied Science), which is a terminal deoxynucleotidyltransferase-mediated dUTP nick-labeling (TUNEL), as in Lu *et al.* (29). Human T cells were incubated with 0.1–10 nM PD1 or vehicle for 48 h at 37 °C. In some experiments the cells were pretreated with nystatin (5 µg/ml) prior to incubation with PD1 or vehicle. The cells then were fixed on slides with 4% paraformaldehyde, immersed in terminal deoxynucleotidyltransferase buffer, and incubated with terminal deoxynucleotidyltransferase buffer and fluorescein-dUTP for 60 min at 37 °C. Next, the slides were washed with PBS, counterstained with propidium iodide and 4,6-diamidino-2-phenylindole, and examined under a fluorescence microscope.

Lipid Raft Patches—For fluorescence microscopy, Jurkat cells were immobilized on glass coverslips coated with 1% (v/v) poly-L-lysine for 30 min. The cells were then incubated with PD1 (0.1–50.0 nM), Fas ligand (2 ng/ml), (18R)-THEPE (10 nM), or DHA (1 µM). In some experiments the cells were pretreated with nystatin (5 µg/ml) before being incubated with PD1 or Fas ligand. After 30 min, the cells were fixed (10 min, 4% paraformaldehyde) and washed. The cells were then stained with anti-CD95 monoclonal antibody, washed 3–5 times with PBS, and then incubated with Texas Red-conjugated F(ab)₂-fragments of rabbit anti-mouse antibodies. The cells were then washed and stained for 45 min with 200 ng/ml FITC-labeled CTX in PBS. Negative control staining was performed with isotype-matched mouse antibodies. Images were obtained with a conventional Zeiss fluorescence microscope with a digital camera or with a Leica TCS NT scanning confocal microscope.

For quantitative analysis, the images of 20 randomly selected cells/slide were examined in a double-blind fashion. The number of positive cells with co-patching of CTX-FITC and Texas Red anti-Fas and the percentage of those cells in the cell population in at least 6–8 independent experiments were expressed as means ± S.E.

Statistics—Results are presented as means ± S.E. The significance of the differences in mean values between and within multiple groups was examined using analysis of variance for repeated measures followed by Duncan's multiple range tests. Student's *t* test was used to evaluate statistical significance of differences between two-paired observations. *p* < 0.05 was considered statistically significant.

RESULTS

PD1 Generation by Human T_{H2}-skewed PBMC Is Dependent on Expression and Activity of 15-Lipoxygenase-1—By using LC-MS-MS lipidomic analysis, we examined whether T_{H2}-skewed PBMC produce PD1 from DHA (Fig. 1), because the expression of 15-LO-1, a major enzyme that appears to be involved in the conversion of DHA to PD1 (15), is induced by T_{H2}-polarizing cytokines, such as interleukin (IL)-4 and IL-13, in alveolar macrophages (30), peripheral blood monocytes (31), and dendritic cells (32). Incubation of T_{H2} PBMC with DHA, but not T_{H1} PBMC, led to the production of the (17S)-hydroxy-containing series compounds (Fig. 1), among which (17S)-hydroxy-DHA ((17S)-HDHA) and PD1 were identified as major products, as well as residual amounts of (14S)-hydroxy-DHA products (Fig. 1A).

(17S)-HDHA was identified (Fig. 1B) from its characteristic MS-MS spectrum, which displayed diagnostic ions at *m/z* 343 (M – H), 325 (M – H – H₂O), 299 (M – H – CO₂), 281 (M – H – H₂O – CO₂), 273

and 255 (273 – H₂O), 245 and 229 (273 – CO₂), and 201 (245 – CO₂); and its retention time and UV spectrum confirmed the presence of a conjugated diene in the structure (an asymmetric peak with a maximal absorbance wavelength [λ_{\max}] at 236 nm) (Fig. 1B, inset). (17S)-HDHA is produced by reduction from its (17S)-hydro(peroxy)-DHA precursor, the major product of 15-LO action with DHA (15), an intermediate that can be converted by further action of 15-LO to PD1, identified in these incubation extracts by its characteristic ions present in its MS-MS spectrum. These results are consistent with the physical properties of NPD1 (15, 16, 18); its MS-MS showed several prominent diagnostic fragment ions at *m/z* 359 (M – H), 341 (M – H – H₂O), 323 (M – H – 2H₂O), 315 (M – H – CO₂), 297 (M – H – H₂O – CO₂), and 277 (M – H – H₂ – 2H₂O – CO₂). Additional diagnostic ions demonstrated the presence of the 10 and 17 alcohol-containing carbon positions, including those at *m/z* 153 and 163 (181 – H₂O); 181, 205, and 217 (261 – CO₂); 227 (289 – H₂O – CO₂); 243 (261 – H₂O); and 261 and 289 (M – H – CH₃CH₂CHCHCH₃). The UV spectrum of this dihydroxydocosatriene in methanol gave a λ_{\max} at 270 nm with shoulders at 261 and 281 nm, a triple band of absorption consistent with the conjugated triene structure present in PD1 (data not shown) (15). The complete stereochemical assignment of NPD1 (15–18), as well as this PBMC-DHA-derived product, was determined by matching materials prepared by total organic synthesis.⁵ NPD1/PD1 generated by human PBMC carried the complete stereochemistry of (10R,17S)-dihydroxydocosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid and was matched to the several dihydroxytriene-containing DHA-derived products isolated from human PBMC. To assess the biosynthesis and to determine whether an epoxide-containing intermediate or alternatively a double dioxygenation was involved in PBMC production of this bioactive product, alcohol trapping experiments were carried out as earlier reported with microglial cells (15). Briefly, methanol trapping of the potential epoxide-containing intermediates during the exposure of DHA to T_{H2} PBMC led to the production and identification of 10-methoxy-17-hydroxy DHA (*n* = 4); diagnostic ions in its MS/MS spectrum were at *m/z* 373, 152, 221, 275, 323, 329, 341, and 355, which were essentially identical to those reported earlier in microglial cells from the brain (15). These results indicate that the main route for formation of bioactive PD1 by PBMC is via an epoxide-containing intermediate (see Fig. 1H for illustration).

It was shown previously that the DHA content in the membranes of lymphoblasts increases during their activation (33). To determine whether T_{H2} PBMC produce PD1 from endogenous DHA, we incubated these cells in the absence of exogenous DHA, and we determined the levels of DHA-derived products formed. We found that T_{H2} PBMC produce PD1 and (17S)-HDHA (0.5–2.4 and 1.1–3.8 ng per 20.0 × 10⁶ cells, respectively). Of interest, we found that addition of IL-4 during the incubation time led to a significant reduction in the production of DHA-derived lipid mediators (Fig. 1C). Both PD1 and (17S)-HDHA levels were reduced (83.5 ± 3.1 and 26.0 ± 6.5% of control, respectively), although the preferential production of PD1 over (17S)-HDHA suggests an important role for this product in the skewing of T_{H2} PBMC.

To examine the expression of putative PD1-generating enzymes, we determined whether 15-LO-1 expression is induced under T_{H1}- or T_{H2}-skewing conditions. These results (Fig. 1D) indicated that T_{H2} PBMC expressed higher levels of the spliced mRNA species of 15-LO-1 (which resulted in a PCR product of ~300 kb) than did T_{H1} cells. T_{H1} PBMC expressed very low levels of spliced 15-LO-1 but significantly

⁵ Serhan, C. N., Gotlinger, K., Hong, S., Lu, Y., Siegelman, J., Baer, T., Yang, R., Colgan, S. P., and Petasis, N. A. (2005) *J. Immunol.*, in press.

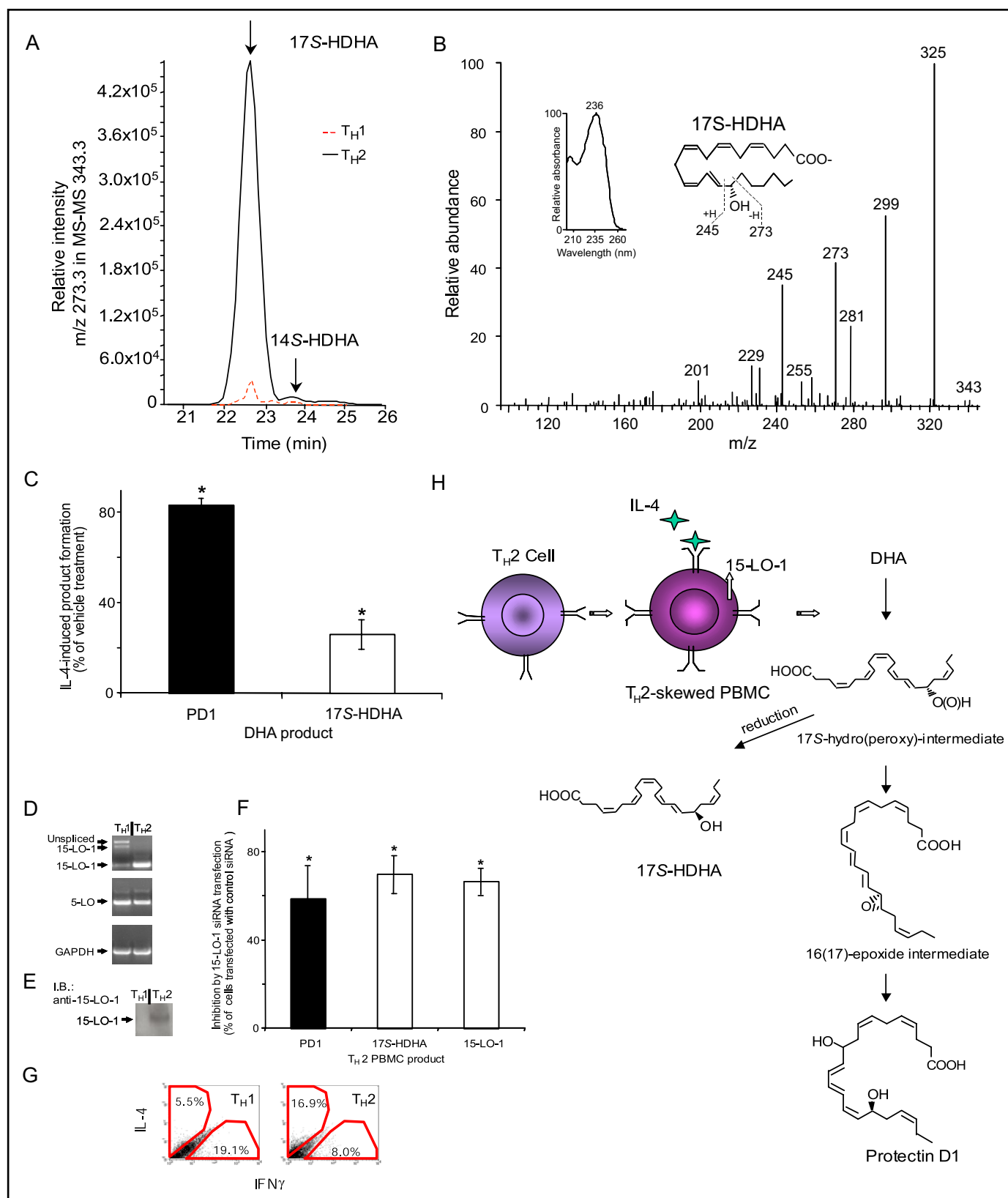


FIGURE 1. Production of PD1 and expression of 15-LO by T_H2 - but not T_H1 -skewed human PBMC. A, select ion chromatogram (at m/z 273.3 in MS-MS 343.3), acquired from LC-UV-MS-MS-based lipidomic analysis, showing biosynthesis of (17S)-HDHA by T_H2 - (solid line) or T_H1 -skewed (broken line) PBMC after incubation with DHA. Results are representative of three experiments. B, MS-MS and UV (inset) spectra of (17S)-HDHA produced by T_H2 -skewed human PBMC. The products were identified on the basis of MS-MS and UV spectra, as well as the chromatographic retention times. Results are representative of three experiments. C, human PBMC were cultured under T_H2 -skewing conditions, and then IL-4 (10 ng/ml) or vehicle was added (60 min, 37 °C). The products were extracted as described under "Experimental Procedures" and analyzed by LC-UV-MS-MS. Results are representative of three experiments. *, $p < 0.05$ (comparing treatments with vehicle and IL-4). D and E, human PBMC were cultured under T_H1 - (left lane) or T_H2 - (right lane)-skewing conditions. Next, their mRNA was used for RT-PCR with specific 5-LO and 15-LO primers (D), and the products were visualized. The protein from these cells was run by SDS-PAGE and blotted with anti-15-LO-1 (E). I.B., immunoblot. Results are representative of four experiments. F, human PBMC were transfected with 15-LO-1 siRNA or control and cultured under

higher levels of the unspliced mRNA species of 15-LO-1 (PCR product of ~1250 kb (34)), which was essentially absent in T_H2 cells. The low level of expression of 15-LO-1 mRNA also was observed in activated but not skewed PBMC that were treated by concanavalin A alone (data not shown; *n* = 3). Of interest, similar levels of PCR products corresponding to the expression of 5-LO mRNA were noted in T_H1 and T_H2 cells (Fig. 1D), but only trace amounts of 4- and 7-HDHA, the putative products of DHA with 5-LO (35), were detected with either cell type. Results were similar for cells collected on days 3 and 6 of culture (data not shown; *n* = 3). In addition, 15-LO-1 protein expression was evident in T_H2 but not in T_H1 PBMC or T cells (Fig. 1E and data not shown), and both PD1 and (17S)-HDHA production was blocked by silencing of 15-LO-1 expression in T_H2 PBMC (58.7 ± 15.0, 69.6 ± 8.5, and 66.3 ± 6.1% inhibition of PD1 and (17S)-HDHA production, and 15-LO-1 expression, respectively; see Fig. 1F). The recently cloned 15-LO type 2 (36) was not apparently expressed in T_H2-skewed PBMC at either the mRNA or protein level (data not shown; *n* = 8). To confirm the skewing toward T_H1 and T_H2 phenotypes, the profile of cytokines produced by the cells was examined. We found that in the T_H1 PBMC, 19.1 and 5.5% of the cells produced IFN γ and IL-4, respectively. In contrast, in the T_H2 PBMC, 8.0 and 16.9% of the cells produced IFN γ and IL-4, respectively (Fig. 1G). Thus, these culturing conditions skewed the PBMC toward T_H1 or T_H2 phenotypes. Taken together, these results indicate that T_H2-skewed PBMC express 15-LO-1 that converts DHA to PD1 (illustrated in Fig. 1H).

PD1 Blocks Leukocyte Inflammatory Migration in Vivo—Lipid mediators generated during the resolution of inflammation, such as lipoxins and resolvins, stop leukocyte infiltration into inflamed sites (4, 27). To determine whether PD1 reduces lymphocyte inflammatory migration, we initiated peritonitis by intraperitoneal administration of zymosan A in mice given an intravenous injection of PD1 (100 ng/mouse) or vehicle alone. Fig. 2A is a typical microphotograph showing leukocytes infiltrating into the peritoneal lining. The tissues of mice exposed to zymosan A showed an abundance of extruded proteins (red staining by eosin) and inflammatory cells (nuclear black staining by hematoxylin). Accompanying the cell and protein infiltration was marked vascular congestion and local tissue edema, the hallmarks of peritonitis (data not shown). Both protein and cellular infiltration were substantially decreased in the peritoneum of mice that received PD1. Staining of cells recovered from the peritoneal lavage with lymphocyte markers (CD3 for T cells and CD19 for B cells) and analyzed by FACSsort revealed (Fig. 2B) that PD1, but not its precursor DHA, given at equimolar concentrations, reduced lymphocyte and T and B cell numbers in the exudates (50.0 ± 8.2, 41.7 ± 11.0, and 49.9 ± 9.9% inhibition, respectively). These results indicate that, in addition to stopping PMN infiltration (15), PD1 potently regulates the migration of inflammatory leukocytes and particularly lymphocytes.

PD1 Inhibits Cytokine Secretion from Human Peripheral Blood T Cells—Our results (Fig. 2) suggested that lymphocytes are one of the main targets of PD1. Next we examined the impact of PD1 on the secretion of cytokines by human peripheral blood T cells because cytokine deprivation *per se* participates in the resolution of inflammation and is regulated by other lipid mediators of resolution, such as lipoxins (28). Antibodies against CD3 and CD28 were used to mimic the stimulation by antigen-presenting cells that leads to the secretion of TNF α and

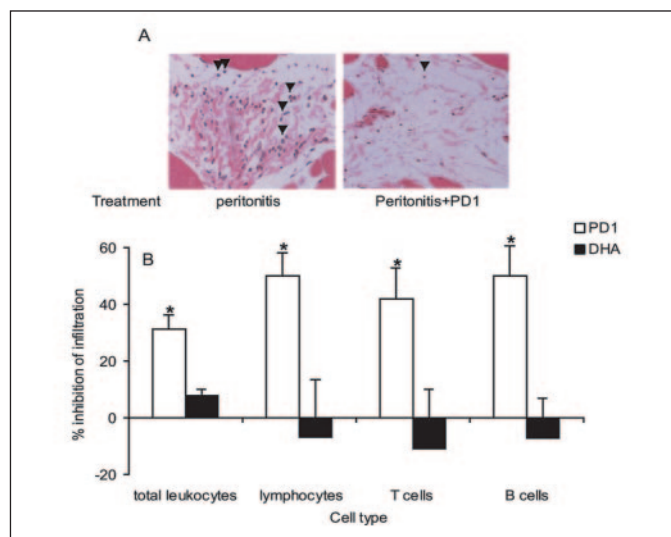


FIGURE 2. PD1-induced inhibition of leukocyte infiltration in zymosan A-induced peritonitis. A, mice were injected intravenously with vehicle (left) or PD1 (100 ng/mouse; right), followed by an intraperitoneal injection of zymosan A (1 mg). After 2 h the peritoneal infiltrate was recovered, and the peritoneal lining was used for histological sections. Infiltrating leukocytes (marked by arrowheads) and protein were detected by hematoxylin and eosin staining. B, mice were injected intravenously with vehicle, DHA, or PD1 (100 ng/mouse; right), followed by an intraperitoneal injection of zymosan A (1 mg). The peritoneal lavage cells were enumerated, stained for lymphocyte markers, and analyzed by FACSsort. *, *p* < 0.05 (comparing treatments with PD1 and DHA). Results are representative of three experiments.

IFN γ by T cells. PD1 inhibited, in a concentration-dependent manner, the secretion of both cytokines induced by anti-CD3 and anti-CD28 (Fig. 3, A and B). At 10 nM, PD1 significantly abrogated the co-stimulation with anti-CD28 (80.1 ± 5.4 and 104 ± 28.6% inhibition of TNF α and IFN γ secretion, respectively; see Fig. 3C). In addition, the minimal concentration of PD1 significantly inhibiting the production of both TNF α and IFN γ was 0.01 nM, although abrogation of IFN γ secretion was greater than that of TNF α secretion at all concentrations (Fig. 3, A and B). A similar impact on IFN γ secretion was obtained when the cells were exposed to synthetic PD1 (data not shown). T cells viability and proliferation following stimulation by anti-CD3 + anti-CD28 were not significantly modulated by PD1 (*n* = 3), indicating that PD1 was not toxic to T cells. Thus, PD1 potently and directly inhibits cytokine secretion by human T cells.

PD1 Promotes Apoptosis of Human T Cells—Because apoptosis substantially contributes to T cell clearance during resolution (37), we sought to determine whether PD1 exerts its anti-inflammatory actions through promotion of T cell apoptosis. We used TUNEL fluorescent microscopy to examine PD1-induced cell death. PD1 markedly increased the percentage of DNA-fragmented apoptotic cells (Fig. 4A). The quantitative results (Fig. 4B) indicate that PD1 induced a significant and concentration-dependent increase in T cell death. The maximal impact was obtained with 10 nM PD1, which resulted in a 5-fold increase in T cell apoptosis (13.2 ± 2.8 and 65.4 ± 3.8% apoptotic cells for vehicle and 10 nM PD1, respectively), indicating that PD1 is a potent promoter of T cell apoptosis. Of interest, PD1 did not induce T cell apoptosis when the cells were activated by antibodies against CD3 and CD28 (*n* = 3), indicating that stimulation of T cells through their TCR overrides the apoptotic signal induced by PD1.

T_H2-skewing conditions as described under "Experimental Procedures" and then incubated with DHA (20 μ M). After 60 min the products were extracted and analyzed by LC-UV-MS-MS. 15-LO-1 expression was determined in these T_H2-PBMC by staining with anti-15-LO-1 antibodies and analysis by FACSsort. Results are representative of three separate experiments. *, *p* < 0.05 (comparing transfection with control and 15-LO-1 siRNA). G, human PBMC were cultured under T_H1- (upper panel) or T_H2- (lower panel)-skewing conditions. After 6 days the cells were collected and activated by anti-CD3 + anti-CD28 for 5 h in the presence of GolgiPlugTM. Then the cells were stained for intracellular IFN γ and IL-4 and analyzed by FACSsort. Results are representative of four experiments. H, schematic illustration of PD1 production by T_H2 PBMC.

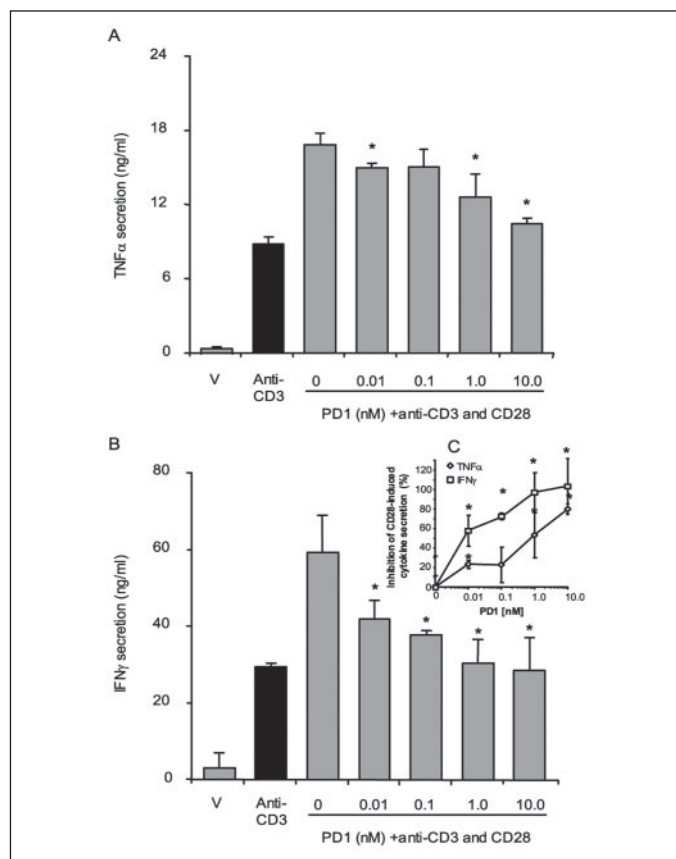


FIGURE 3. PD1 inhibits TNF α and IFN γ secretion by human T cells. A and B, human peripheral blood T cells were incubated with 0.01–10.0 nM PD1 or vehicle (0.05% EtOH) for 6 h and then incubated with anti-CD3 or anti-CD3 + anti-CD28 for 41 h as indicated. The supernatants were collected, and the levels of TNF α (A) and IFN γ (B) were determined by standard enzyme-linked immunosorbent assay. C, the calculated inhibition of anti-CD28 co-stimulation by PD1. *, $p < 0.05$ versus treatment with anti-CD3 + anti-CD28. Results are representative of five experiments.

Formation of lipid raft clusters represents one of the earliest responses to apoptotic factors such as Fas ligand and plays a pivotal role in transduction of the apoptotic signal (38). To explore the mechanism by which PD1 induces T cell apoptosis, we examined whether PD1 activates lipid raft clustering on T cell membranes and thereby initiates apoptosis. The clustering of membrane lipid rafts was measured in Jurkat T cells with staining of GM1 gangliosides with FITC-labeled CTX and detection with confocal microscopy, as described under the “Experimental Procedures” (Fig. 5A). The patches of FITC-labeled CTX and ganglioside complex represented the clusters of lipid rafts. Treatment with vehicle resulted in lipid raft distribution throughout the T cell membrane, as indicated by a very weak green diffused FITC fluorescence (Fig. 5A, vehicle in *middle panel*). Green fluorescence cap or patches on the cell membrane were detected in cells that were exposed to 10 nM PD1 or Fas ligand at 2 ng/ml for 30 min. These results clearly showed that lipid rafts were aggregated into a large plasmalemmal patch. Of interest, CD95 (or Fas) was also aggregated on the cell membrane in a similar pattern when these T cells were incubated with PD1 (Fig. 5A, *left panels*). The overlay of the two is depicted by *yellow* areas (Fig. 5A, *right panels*) that represent co-localization of Fas and CTX-labeled gangliosides (lipid raft cluster). It is clear that the lipid raft platforms are formed in response to PD1 or Fas ligand in human T cells. This lipid raft clustering evoked by PD1 may be associated with Fas aggregation and activation.

As shown in Fig. 5B, PD1 significantly increased the formation of lipid raft clusters in a concentration-dependent manner from $6.8 \pm 2.4\%$ for

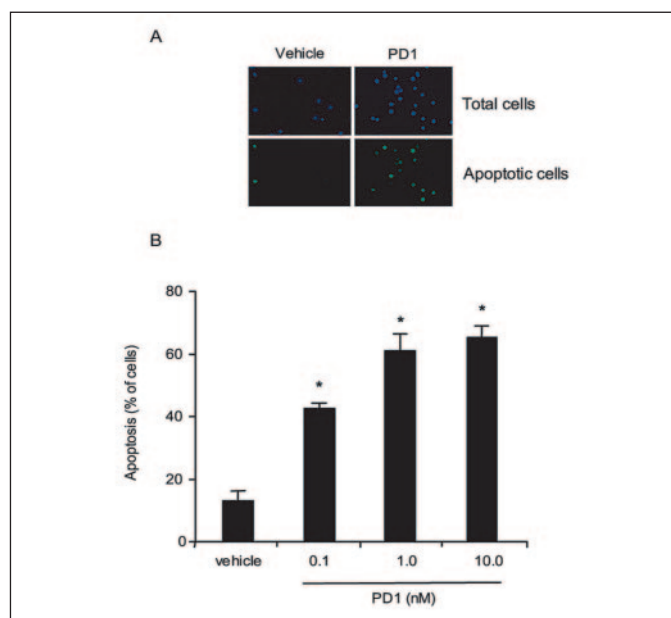


FIGURE 4. PD1 promotes human T cell apoptosis. A, human T cells were incubated with vehicle or PD1 (10 nM) for 48 h and then analyzed for apoptosis by TUNEL. The stained cells were photographed (all cells, *upper panels*; apoptotic cells, *lower panels*). B, human T cells were incubated with vehicle or 0.1–10.0 nM PD1 for 48 h and then analyzed for apoptosis by TUNEL. The stained cells were counted, and the percentage of positive cells was calculated. *, $p < 0.05$ versus vehicle. Results are representative of four separate experiments.

vehicle to $61.3 \pm 1.7\%$ at 10 nM PD1. Similar results were obtained with peripheral blood T cells following incubation with 0.1–10 nM of PD1 (Fig. 5C). This increase in the formation of lipid raft clusters induced by PD1 and Fas ligand was significantly blocked (50.1 ± 4.0 and $64.2 \pm 5.0\%$ inhibition of PD1- and Fas ligand-induced clustering, respectively) by nystatin, a membrane cholesterol sequestration reagent (39), indicating that the detected fluorescent patches are specific lipid raft clusters.

To determine the specificity of PD1 on lipid raft cluster formation and Fas aggregation, we tested the impact of functionally and structurally related compounds such as native DHA, ATLa, and (18R)-THEPE. Native DHA itself at concentrations of 1 μM moderately increased the formation of lipid raft clusters from 7.6 ± 1.1 to $21.6 \pm 4.4\%$, which was less than that obtained with PD1 at concentrations as low as 0.1 nM. Hence it is possible that the actions observed for DHA may be accounted for by its conversion to PD1 by these cells. Of interest, neither (18R)-THEPE nor ATLa had a significant effect on the formation of lipid raft clusters (data not shown). To examine whether PD1-induced apoptosis is dependent on lipid raft formation, we treated human T cells with nystatin, PD1, or both agents and determined cellular apoptosis. Nystatin completely blocked PD1-induced apoptosis ($95.6 \pm 4.5\%$ inhibition) (Fig. 5D), whereas nystatin alone slightly promoted cell death. Taken together, our results indicate that PD1 is a potent inducer of lipid raft clustering accompanied by Fas aggregation in these rafts and that this process mediates the activation of a death signal and PD1-evoked apoptosis (Figs. 4 and 5).

DISCUSSION

In the present study, we report that a novel docosatriene, PD1, is produced by T_H2 PBMC, that this production is accompanied by increased expression of 15-LO-1 in T_H2 but not T_H1 PBMC, and is dependent on 15-LO-1 activity. We also found that PD1 is a potent inhibitor of T cell infiltration to zymosan A-treated peritoneal cavities and of cytokine secretion by TCR-stimulated human T cells, and we

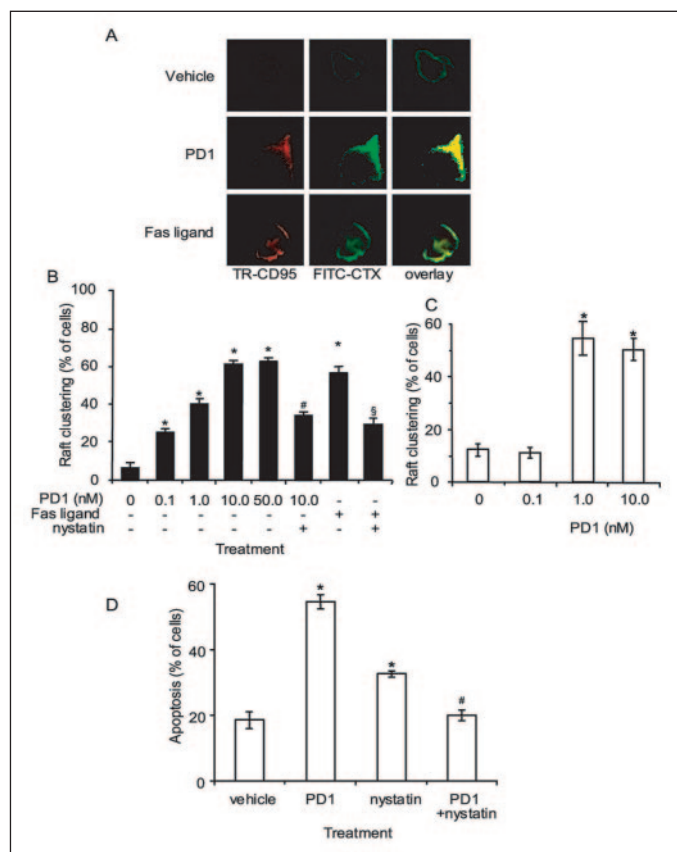


FIGURE 5. PD1 promotes formation of lipid raft clusters and aggregation of Fas that lead to apoptosis in human T cells. A, Jurkat cells were incubated with vehicle, PD1 (10 nM), or Fas ligand (2 ng/ml) for 30 min and then stained with Texas Red-conjugated mouse anti-Fas monoclonal antibody (left panels) and FITC-conjugated CTX (middle panels). The two types of staining were superimposed on each other (right panels). B, Jurkat cells were incubated with vehicle, PD1 (0.1–50.0 nM), or Fas ligand (2 ng/ml) or with nystatin (5 μ g/ml) and PD1 (10 nM) or Fas ligand (2 ng/ml), stained as indicated above, and counted for percentage of positive cells. *, $p < 0.05$ versus vehicle. #, $p < 0.05$ versus Fas ligand. Results are representative of four experiments. C, human peripheral blood T cells were incubated with PD1 (0.1–10.0 nM), stained as indicated above, and counted for percentage of positive cells. *, $p < 0.05$ versus vehicle. Results are representative of three experiments. D, human T cells were incubated with vehicle, PD1 (10 nM), nystatin (5 μ g/ml), or both compounds for 48 h and then analyzed for apoptosis by TUNEL assay. *, $p < 0.05$ versus vehicle. #, $p < 0.05$ versus 10 nM PD1. Results are representative of at least six experiments.

found that PD1 promotes T cell apoptosis via the formation of lipid raft clusters. The immunoregulatory properties of the recently identified DHA-derived mediator, denoted earlier as (10,17S)-DT (15) or in neuronal systems as NPD1 (16), that were revealed in this study led us to propose PD1 to denote the wide scope of its formation and actions. Glimcher and co-workers (14, 40) showed earlier that favoring the development of T_H2 cells leads to suppression of inflammation and autoimmune disorders *in vivo*, particularly in DHA-enriched tissues such as the brain. In addition, the findings of Hibbeln (9) and Moriguchi and Salem (10) that indicate a substantial role for DHA in neural development and function raise the likelihood that DHA serves as an essential substrate/precursor in the assembly and maintenance of neural tissue. Indeed, the infiltration of PMN into the brain during stroke is regulated by the production of PD1, which protects it from PMN-inflicted neural tissue injury (16). Also, in recent studies PD1 was found to be produced during the course of a murine model of asthma, a well established T_H2 -generated disorder.⁶ Administration of PD1 blocked

both eosinophil and lymphocyte infiltration to airways as well as proinflammatory mediator production following aerosol challenge. Our present results establish that 15-LO-1 expression and activity is up-regulated specifically in human T_H2 but not in T_H1 PBMC (Fig. 1), resulting in the production of docosatrienes by these cells. Of interest, silencing of 15-LO activity resulted in substantial inhibition of both PD1 and (17S)-HDHA generation. Moreover, it has been shown recently that 12/15-LO-deficient mice had a defect in both re-epithelialization and PMN recruitment in the cornea, and both functions were enhanced by NPD1 as well as lipoxin A_4 (41), and that 15-LO-1 and NPD1/PD1 levels were reduced in the hippocampal cornu ammonis region 1 of Alzheimer disease patients (42). These findings support our observations in attributing a role for 15-LO-1 in the NPD1/PD1 generation. Hence, PD1, a novel resolution mediator (15), can be produced by a T_H2 -skewed neural system, where infiltrating T_H2 cells will impose 15-LO-1 expression that could exploit the DHA-enriched environment. Consequently, PD1 will contribute to the suppressive impact of T_H2 cells on brain inflammation.

TNF α and IFN γ produced by T cells are critical for activation of monocytes and the establishment of chronic inflammatory disorders such as arthritis, inflammatory bowel diseases, and other conditions (43–45). The deprivation or withdrawal of these cytokines is implicated in the resolution of inflammation (20, 25). PD1 potentially inhibited the production of both TNF α and IFN γ by T cells stimulated by anti-CD3 + anti-CD28 (Fig. 3). These results are in agreement with a role for PD1 as a T_H2 mediator, because it counteracts the production of IFN γ , a major T_H1 cytokine. T cell clearance during resolution of inflammation is associated with the apoptosis of activated cells, namely activation-induced cell death (37). We report that incubation of human T cells with PD1 markedly increased cell death (Fig. 4). The results provide the first direct evidence that PD1 can induce T cell apoptosis. As a potent inducer of T cell apoptosis, this docosatriene may significantly increase the clearance of T cells in the local inflammatory microenvironment and thereby result in the resolution of inflammation.

Recent studies have indicated that activation-induced cell death is mediated by interactions of Fas and its ligand on T cells (46). In the present experiments, we found that PD1 induced T cell apoptosis. Therefore, we examined whether PD1 induces apoptosis of T cells through a pathway similar to that of Fas activation. Lipid rafts are referred to as the microdomains on cell plasma membranes, which are enriched in sphingolipids and cholesterol and resistant to solubilization by nonionic detergents (47). When trimeric Fas ligand binds to Fas, the aggregation of Fas initiates a signaling cascade that leads to cell apoptosis (38, 48). Fas associates with lipid rafts on T cell membranes (38), and Fas-related functions in these cells are dependent on its clustering in cholesterol-containing lipid rafts, which stabilize the Fas death-inducing signaling complex. Agents that block lipid raft clustering completely abolish Fas ligand-induced cell apoptosis (38). PD1 stimulated clustering of lipid rafts on the T cell membrane to an extent similar to that induced by Fas ligand (Fig. 5). Of interest, the actions of PD1 on this lipid raft clustering on T cell membranes are accompanied by Fas aggregation rather than by an increase in Fas expression, because Western blot analysis demonstrated that Fas protein expression by T cells was not increased by exposure to PD1 (data not shown). These results suggest that PD1 may serve as an activator of a Fas-like death signaling.

A broad overview of the impact of DHA in a variety of pathophysiologic conditions ranging from inflammatory and autoimmune disorders to neural development and cardiac protection (5, 7, 49) underscores the beneficial properties of DHA as a supplementary nutrient. However, the mechanism of DHA actions is still not fully defined in molecular terms.

⁶ B. D. Levy, P. Kohli, K. Gotlinger, S. Hong, K. J. Haley, and C. N. Serhan, submitted for publication.

DHA Pathways and Actions of Protectin D1 with Human PBMC

The present results demonstrated that PD1, a novel product of newly described DHA-derived pathways, is produced by a T_H2-skewed environment, reduces T cell migration to an inflamed microenvironment *in vivo*, and reduces the production of cytokines. PD1 also promoted apoptosis in human T cells, which was associated with lipid raft clustering. Thus, PD1 is a potent regulator of T cell inflammatory functions and might play a role in the anti-inflammatory, developmental, and protective properties attributed to ω -polyunsaturated fatty acids (*i.e.* DHA) and in directly regulating the suppressive action of T_H2 cells in the inflammation of the central nervous system. These findings could lead to the development of new therapeutic approaches to treat T cell-mediated inflammation and autoimmunity.

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The Docosatriene Protectin D1 Is Produced by T_H2 Skewing and Promotes Human T Cell Apoptosis via Lipid Raft Clustering
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