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The *Pseudomonas aeruginosa* Quorum-Sensing Molecule *N*-3-(Oxododecanoyl)-L-Homoserine Lactone Inhibits T-Cell Differentiation and Cytokine Production by a Mechanism Involving an Early Step in T-Cell Activation

A. J. Ritchie,[1] A. Jansson,[1,2] J. Stallberg,[1] P. Nilsson,[2] P. Lysaght,[1] and M. A. Cooley[1]*

School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, Australia,[1] *and Computational Biology, Department of Life Science, University of Skövde, Skövde, Sweden*[2]

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The *Pseudomonas aeruginosa* quorum-sensing molecule *N*-3-(oxododecanoyl)-L-homoserine lactone (OdDHL) has been reported to have immunomodulatory activity in several systems, although the mechanism of that activity remains to be fully characterized. We demonstrate here, using a defined in vitro model of antigen responses by T-cell receptor (TCR)-transgenic mouse splenic CD4 T cells, that the effect of OdDHL on activation and cytokine production is complete within 4 h of antigen or mitogen stimulation and does not depend on the insertion of OdDHL in the cell membrane, despite a previous report that immunosuppression by homoserine lactones required a minimum acyl chain length of 11 carbons (S. R. Chhabra, C. Harty, D. S. W. Hooi, M. Daykin, B. W. Bycroft, P. Williams, and D. Pritchard, J. Med. Chem. 46:97-104, 2003). We also demonstrate that while OdDHL can have toxic effects on nonlymphoid leukocytes, it does not induce significant cell death in T cells at the concentrations (≤10 μM) used in these experiments. In addition, we show that primary and secondary antigen-specific cytokine responses are equally susceptible to inhibition by OdDHL and that the compound inhibits the differentiation of both Th1 and Th2 cells. However, the precise balance of cytokine production by CD4 T cells stimulated in the presence of OdDHL varies with both the antigen concentration and its affinity for the transgenic TCR. Thus, conflicting reports of the nature of the immunosuppression by OdDHL may be due in part to the differences in antigen affinity and concentration in different models.

The *Pseudomonas aeruginosa* quorum-sensing molecule *N*-3(oxododecanoyl)-L-homoserine lactone (OdDHL) has been observed by several groups to affect the functional activities of a range of mammalian cell types, including respiratory epithelial cells (6, 12, 16), fibroblasts (16, 19), arterial smooth muscle cells (8, 10), and cells of the immune system, such as monocytes (19, 20), neutrophils (19), and lymphocytes (4, 14, 17, 20). Our laboratory and others have reported that the in vitro effects of OdDHL on lymphocytes are suppressive, including inhibition of proliferation (4, 20) and cytokine production (14, 17, 20). Other groups have reported that at high concentrations, OdDHL can cause up-regulation of proinflammatory cytokines, such as interleukin 8 (IL-8), in vitro in respiratory epithelial cells (12, 16). Telford et al. suggested that OdDHL has a more pronounced inhibitory effect on Th1-type responses than on Th2-type responses, meaning that OdDHL exposure could lead to a selective down-regulation of proinflammatory responses and a favoring of Th2-type responses (20). Our results in an in vivo model of antigen-specific responses suggested that the effect of OdDHL on immune responses depended on the model chosen, with the net effect being an increased skewing of immune responses already biased toward Th1 or Th2 (14).

The mode of action of OdDHL in mammalian cells remains uncertain. Williams et al. have recently demonstrated that OdDHL can enter mammalian (COS) cells and can function to activate a LasR construct with which these cells have been transfected (21). Smith et al. suggested that at high concentrations (100 μM) it increased activation of NF-κB in respiratory epithelial cells, but they were unable to identify its binding target (16). The activation of NF-κB in T cells is not consistent with the observed inhibition of proliferation and cytokine production (9), suggesting that some other mechanism is operative in T cells. OdDHL is a small molecule consisting of a 12-carbon saturated acyl side chain with a homoserine lactone ring. Work by Chhabra et al. (4) showed that inhibition of concanavalin A-stimulated murine spleen cell proliferation by a range of OdDHL-related compounds required an acyl side chain a minimum of 11 carbons in length and high lipophilicity of the molecule. The homoserine lactone ring was also shown to be essential for efficient suppression of lymphocyte proliferation (4). This raised the possibility that OdDHL acts on mammalian cells by intercalating to the plasma membrane, thereby inhibiting the aggregation of important cell surface receptors, such as the T-cell receptor (TCR).

* Corresponding author. Mailing address: School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, NSW 2052, Australia. Phone: 61-2-93853761. Fax: 61-2-93851591. E-mail: m.cooley@unsw.edu.au.

To investigate the site of action of OdDHL in T cells, we therefore chose to explore the effects of OdDHL in a defined in vitro antigen-specific T-cell response, using a well-described TCR-transgenic mouse model in which the transgenic TCR expressed on the majority of CD4 T cells recognizes a defined peptide antigen from moth or pigeon cytochrome *c* (2); for this model, the way in which the CD4 T-cell-mediated immune response varies with antigen affinity and concentration has been well described (5, 15). The cytokines gamma interferon (IFN-γ) and IL-4 were selected for analysis as markers of type 1 (Th1) and type 2 (Th2) effector CD4 T-cell responses (11). Using this model, and by comparing both antigen-specific and mitogenic (ionomycin plus phorbol myristate acetate [PMA]; anti-CD3ε) stimulation, we demonstrate here that the effects of OdDHL on T-cell cytokine production are not due to toxicity or death of T cells, that OdDHL must be present within the first 2 h after antigen or mitogen stimulation to cause inhibition of T-cell function, and that its site of action is likely to be cytoplasmic rather than membrane. In addition, we demonstrate that the effect of OdDHL on CD4 T cells is to inhibit the differentiation of both Th1 and Th2 cells and that the precise effect of OdDHL can be modulated by antigen affinity.

MATERIALS AND METHODS

Mice. Specific-pathogen-free TCR-transgenic mice on a B10.BR genetic background that expressed a TCR specific for the COOH-terminal epitope of moth cytochrome *c* (MCC) on 50 to 80% of peripheral $CD4^+$ T cells (2) were bred in house from homozygous breeding pairs kindly provided by B. Fazekas de St Groth. All mice used were males, 8 to 12 weeks old. Specific-pathogen-free 8- to 12-week-old male B10.BR mice were purchased from the Animal Resource Centre (Canningvale, Australia). The mice were housed in accordance with National Health and Medical Research Council of Australia guidelines and given sterile food and water ad libitum. All animal experiments were approved by the University of New South Wales Animal Care and Ethics Committee (ACEC00/01, ACEC02/68, and ACEC03/10).

OdDHL. OdDHL was synthesized by Naresh Kumar, School of Chemical Sciences, University of New South Wales, according to the method described by Bycroft et al. (3). The resulting OdDHL was checked for identity and purity by nuclear magnetic resonance, and its activity as a quorum-sensing molecule was confirmed by the quorum-sensing bioassay as described by Fuqua and Winans (7). OdDHL was stored as a stock solution in dimethyl sulfoxide (DMSO) at −70°C and diluted to the required concentration in cell culture medium (CCM) (10% [vol/vol] heat-inactivated fetal calf serum [Trace Bioscience, Castle Hill, Australia], 2 mM L-glutamine [Gibco BRL, Gaithersburg, Md.], 50 U of penicillin-streptomycin [Gibco BRL]/ml in RPMI 1640 [Gibco BRL]) immediately before use. For experiments assessing the stability of OdDHL under cell culture conditions, OdDHL was prepared in CCM as described above and then incubated in tissue culture wells for up to 4 h at 37°C, with aliquots removed at 90 min and 4 h. The OdDHL in the CCM was then removed from the wells and added to cells plus antigen in new wells. The residual OdDHL attached to the culture wells was tested by adding 1 ml of culture medium to preincubation wells, mixing it, removing an aliquot for the quorum-sensing bioassay, and adding the cells plus antigen to test immune suppression.

Spleen cell cultures. Single-cell suspensions were prepared from individual mouse spleens, except where otherwise indicated, and erythrocytes were removed by incubation in red cell lysis solution (1.5 M NH_4Cl [Ajax Chemicals], 100 mM $NaHCO_3$ [Ajax Chemicals], 10 mM disodium EDTA [Sigma, Sydney, Australia], pH 7.2) for 12 min, followed by resuspension in CCM. All splenocyte cultures were incubated at 37°C in 5% CO_2 at a concentration of 10^6 cells/ml. The cells were incubated in either 2-ml volumes of CCM in 24-well tissue culture plates (Greiner GmbH, Maybachstrasse, Germany) or 10-ml volumes of CCM in 50-ml tissue culture flasks (Greiner GmbH). Preliminary experiments showed no significant difference in cytokine production by T cells cultured under either set of conditions (data not shown).

Analysis of effects of OdDHL on cell viability. Spleen cells were prepared as described above, resuspended at 10^6/ml, and incubated for 2 h at 37°C with various concentrations (1 to 10 mM) of OdDHL in 0.05% DMSO or DMSO alone. At the end of this period, the cells were washed and labeled with fluorescein isothiocyanate (FITC)-conjugated anti-CD3, anti-CD4, or anti-B220 antibodies (B-D Pharmingen, San Diego, Calif.) for 15 min on ice. After one wash, the cells were resuspended in CCM to which 1 μg of propidium iodide/ml was added. The cells were then analyzed by flow cytometry for percent staining with each stain.

Preparation of purified $CD4^+$ T cells. Single spleen cell suspensions were prepared as described above, but without the erythrocyte lysis, using pooled spleens from groups of four or five TCR-transgenic mice. The cells were resuspended in CCM, and 3 ml of cell suspension was layered onto 3.5 ml of Ficoll-Paque PLUS (Amersham Biosciences AB, Uppsala, Sweden) and centrifuged at 400 × *g* for 30 min. The interface layer between the Ficoll-Paque PLUS and CCM layers was collected, washed, and resuspended in 10 ml of CCM. The cells were incubated in 50-ml tissue culture flasks at 37°C in 5% CO_2 for 1 h, followed by collection of nonadherent cells, which were labeled for 15 min at 4°C with anti-mouse CD4 MACS microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions, and separated on an LS+ MACS column (Miltenyi Biotec GmbH) on a varioMACS (Miltenyi Biotec GmbH). The resultant $CD4^+$ fraction was assessed for purity by flow cytometry using anti-CD4–FITC (B-D Pharmingen), and only populations that were >95% $CD4^+$ were used in further experiments. For experiments inwhich purified TCR-transgenic $CD4^+$ T cells were stimulated with antigen, splenic antigen-presenting cell (APC) populations were prepared from pooled B10.BR spleens as described above, followed by 2,500 rads of irradiation using a ^{137}Cs source.

Stimulation of T cells. Splenocytes or purified $CD4^+$ T cells from TCR-transgenic mice were stimulated with either wild-type MCC peptide or the MCC variant peptide T102S (MCC_{T102S}) (Auspep, Parkville, Australia) at concentrations ranging from 0.01 to 5 μM as indicated for individual experiments. In some experiments, spleen cells were stimulated with mitogenic anti-CD3ε (145-2C11; B-D Pharmingen) at 5 μg/ml or with 1 mg of Ca^{2+} ionophore (ionomycin; Calbiochem)/ml plus 8 ng of PMA (Sigma)/ml. For ionomycin-PMA stimulation, cells were incubated in CCM with ionomycin plus PMA, with or without OdDHL, in 0.05% DMSO or in 0.05% DMSO alone at 37°C for 8 h, centrifuged, and resuspended at the same concentration in fresh CCM containing PMA and OdDHL in 0.05% DMSO or in 0.05% DMSO alone for the remainder of the culture period, as preliminary experiments had shown that prolonged incubation in ionomycin leads to cell death. For all stimuli, cultures were performed either in the presence of 0.01 to 10 μM OdDHL in 0.05% DMSO or in the presence of 0.05% DMSO alone for times ranging from 48 h to 6 days, as indicated for individual experiments. Supernatants were harvested from all cultures for measurement of cytokine production by enzyme-linked immunosorbent assay (ELISA).

Primary and secondary stimulation of T cells. Cultures of unfractionated TCR-transgenic spleen cells, or of transgenic $CD4^+$ T cells plus irradiated B10.BR splenic APCs, were set up in both 24-well tissue culture plates and 50-ml tissue culture flasks as described above. Negative control cultures were incubated in CCM only, while all other cultures were stimulated with 0.01 to 5 μM wild-type MCC or MCC_{T102S} in the presence of 0.05% DMSO or 0.05% DMSO and 5 μM OdDHL. Cultures in 24-well tissue culture plates were incubated for 48 h before harvest of supernatants for IFN-γ and IL-4 analysis by ELISA as a measure of primary responses. Cultures in 50-ml tissue culture flasks were incubated for 72 h before being split two- to threefold, with volumes being made up with fresh CCM. Cells in negative control flasks were incubated again in CCM only. Flasks containing cells that were antigen stimulated in the presence of 0.05% DMSO were incubated in CCM and 0.05% DMSO, while flasks containing cells that were antigen stimulated in the presence of 5 μM OdDHL were incubated either with CCM alone or with 0.05% DMSO and 5 μM OdDHL, as indicated for individual experiments. These flasks were incubated for a further 72 h. Cells from all cultures were then harvested and washed twice in CCM to remove residual OdDHL and DMSO. Unstimulated cells were discarded at this stage, as there were insufficient viable cells to continue. The cells were then counted and set up in 2-ml volumes of CCM in 24-well tissue culture plates at a concentration of 10^5 viable cells/ml, to which 10^6 irradiated B10.BR spleen cells were added as a source of fresh APCs, together with 5 μM wild-type MCC peptide or MCC_{T102S} and 0.05% DMSO and with or without 5 μM OdDHL. Negative control wells contained primed T cells and APCs in CCM only, T cells and 5 μM MCC_{T102S} only, or APCs and 5 μM MCC_{T102S} only. The cultures were incubated for a further 24 or 48 h at 37°C in 5% CO_2, and the supernatants were harvested for analysis of IFN-γ and IL-4 by ELISA.

Analysis of cytokine production. IFN-γ and IL-4 levels in cell culture supernatants were analyzed using ELISA Minikit development systems (Endogen, Woburn, Mass.) and the following procedure. Ninety-six-well microtiter plates

(Maxisorp; Nalge Nunc International Corp., Naperville, Ill.) were coated with 100 μl of the appropriate capture antibody/well at 50% of the manufacturer's recommended concentration for IFN-γ plates and 100% of the recommended concentration for IL-4 plates and incubated at room temperature overnight. The plates were blocked at room temperature for 1 h with 200 μl of 2% (wt/vol) bovine serum albumin (BSA; Sigma)/well in Dulbecco's Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, pH 7.2 (PBS), and then washed three times with wash buffer (0.2% polyoxyethylene-sorbitan monolaurate [Tween-20; Sigma] in PBS). Appropriately diluted samples and standards in 2% (wt/vol) BSA in PBS were added and incubated overnight. The plates were washed three times, and 100 μl of the appropriate biotin-labeled detection antibody/well was added at 50% of the recommended concentration for IFN-γ plates and at 100% of the recommended concentration for IL-4 plates. The plates were incubated for 1 to 4 h at room temperature and then washed three times. A 1:6,400 dilution of streptavidin-horseradish peroxidase (100 μl/well; Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) in 2% (wt/vol) BSA in PBS was added, and the plates were incubated for 45 min at room temperature. The plates were washed three times, and 100 μl of ELISA substrate (0.0125% [wt/vol] tetramethylbenzidine [Sigma], 0.02% [vol/vol] hydrogen peroxide [MERCK Pty. Ltd.], 3.6% methanol [Asia Pacific Specialty Chemicals Ltd., Seven Hills, Australia])/well in 0.1 M citrate acetate (0.1 M citric acid [MERCK Pty. Ltd.], 0.1 M sodium acetate [Ajax Chemicals], pH 6.0) was added and developed in the dark for 20 to 60 min before the addition of 100 μl of 0.2 M sulfuric acid (MERCK Pty. Ltd.)/well. The absorbance of samples and standards at 450 nm was read using a Benchmark Microplate Reader (Bio-Rad Laboratories, Hercules, Calif.), and the concentrations of IFN-γ and IL-4 were calculated using Microplate Manager version 5.0.1 (Bio-Rad Laboratories) and Prism version 3.00 for Windows (GraphPad Software, San Diego, Calif.).

Quorum-sensing bioassay. Assays of quorum-sensing activity were performed using an *Agrobacterium tumefaciens* bioassay as previously described (13). *A. tumefaciens* strain A136, which carries a *lacZ* fusion to *traI* and produces a blue color in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Promega) in response to acylhomoserine lactones (7), was inoculated into 1.2% agar medium containing a final concentration of X-Gal and poured into petri dishes in 20-ml volumes. Once set, two wells were punched in each plate, and 30 μl of sample or standard was added in duplicate. The plates were incubated for 24 h at 30°C before the diameters of the developed blue zones were measured as an indicator of quorum-sensing activity.

RESULTS

OdDHL affects early events in T-cell activation. Although Smith et al. (16) suggested that in respiratory epithelial cells, OdDHL acted to stimulate the activation of NF-κB transcription factor, such a mode of action is less likely in T cells, as it is inconsistent with our observations of the inhibitory effect of OdDHL on T-cell cytokine production. Thus, while the site of action in T cells remains uncertain, one strategy to narrow down the possible range of binding targets for OdDHL is to analyze the kinetics of its effect on both antigen-stimulated and mitogen-stimulated CD4 T-cell cytokine production. To do this, TCR-transgenic spleen cells were stimulated with anti-CD3ε antibody or antigen-pulsed splenic APCs in vitro, and total cytokine production in the cultures at 48 h was determined by ELISA. The overall time course of IL-4 and IFN-γ production in this model is shown in Fig. 1, which demonstrates that the bulk of cytokine secretion occurs after 24 h, despite the fact that our previous data indicated that mRNA for both these cytokines is detectable at 4 h (14). To determine the point during activation of T cells at which OdDHL has its effect, 5 μM OdDHL (in 0.1% DMSO) or 0.1% DMSO alone was added to cultures at times from −2 to +16 h of stimulation, where 0 is the time at which anti-CD3 (Fig. 2A) or antigen (Fig. 2B) was added. Supernatants from all cultures were harvested 48 h after stimulation and assayed for IL-4 and IFN-γ by ELISA. IFN-γ only is shown for anti-CD3 stimulation, as this stimulus induced negligible levels of IL-4. From these data, it is clear that OdDHL must be present either before or during the first 2 h after antigen- or mitogen-mediated ligation of the TCR in order to significantly suppress cytokine production by these CD4 T cells. If added 4 h or more after stimulation, OdDHL has no significant effect on total cytokine production. This also confirms previous observations indicating that OdDHL does not have nonspecific toxic effects on CD4 T cells: if that were the case, one would expect a reduction in cytokine production if OdDHL were added at any time within the first 24 to 36 h, when the rate of cytokine protein production is highest.

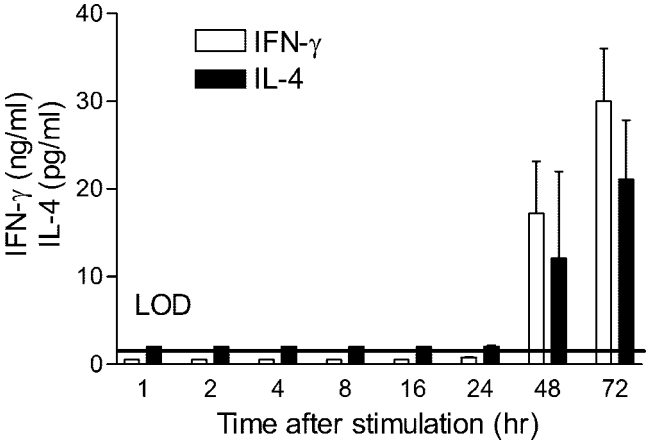

FIG. 1. Time course of cytokine production after stimulation of TCR-transgenic spleen cells with MCC_{T102S} peptide. Cytokine production at each time point was assayed by ELISA. The data are expressed as means plus standard errors of the mean for groups of 10 individual mice. LOD, limit of detection for ELISA (3 pg/ml for IL-4; 3 ng/ml for IFN-γ), represented by the solid line.

Immunomodulatory activity and quorum-sensing activity of OdDHL are linked. While it is clear from a number of publications that OdDHL retains immunomodulatory activity for some time in cell culture (14, 17, 20), it has also been reported to undergo lactonolysis at a pH and temperature similar to those of mammalian cell cultures (37°C; pH 6 to 7) (22). There is also a question of whether OdDHL, being lipophilic and hydrophobic, might bind to the plastic of the wells and thus be unevenly distributed in cultures. For this reason, we incubated OdDHL in cell culture medium in wells of tissue culture plates under culture conditions for up to 4 h. This time frame was chosen since, as shown above, the immunomodulatory effects of OdDHL occur within 4 h of T-cell stimulation. Aliquots of the OdDHL-containing medium were taken at 90 min and 4 h for estimation of quorum-sensing activity in *A. tumefaciens* bioassays (preincubated OdDHL). After 4 h, OdDHL-containing medium was transferred to fresh wells containing splenocytes and antigen peptide. CCM was added to the original wells and mixed, and a small aliquot was taken for assay of quorum-sensing activity (residual OdDHL) before the addition of splenocytes plus antigen peptide to measure residual immunosuppressive activity. Freshly prepared OdDHL was added to other cultures of splenocytes plus peptide in the usual fashion (fresh OdDHL). The results of this experiment are shown in Table 1. After 90 min of incubation at 37°C in CCM, 70 to 80% of the quorum-sensing activity remained, and after 4 h, 40 to

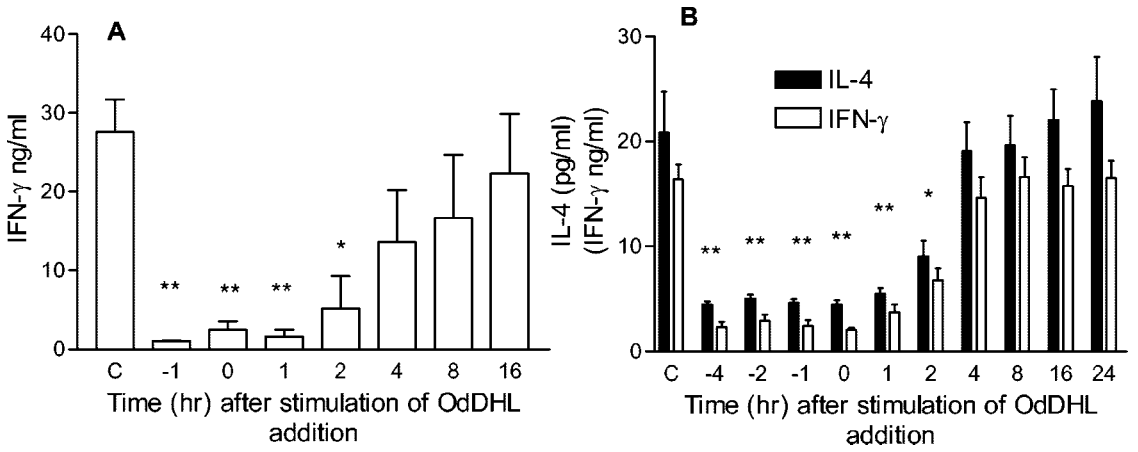

FIG. 2. Effect of time of addition of OdDHL on cytokine production by TCR-transgenic spleen cells stimulated with or anti-CD3ε monoclonal antibody (A) or MCC_{T102S} peptide in both cases, OdDHL was added to the cultures at the times indicated, and the supernatants were harvested 48 h after stimulation (time zero) for cytokine assay. All results are expressed as means plus standard deviations of a minimum of three (A) or nine (B) replicates. Panel A shows IFN-γ only, since anti-CD3 stimulation induces minimal amounts of IL-4 in this model. Statistical analysis by unpaired *t* test compared to DMSO-treated controls (C): **, $P < 0.01$; *, $P < 0.05$.

50% remained. This activity was not attached to the plastic wells but remained in the medium. The degree of inhibition of cytokine production observed in all cultures correlated well with the amount of quorum-sensing activity remaining at the time of T-cell stimulation, suggesting that the two activities require similar degrees of integrity of the OdDHL molecular structure.

OdDHL can act on intracellular targets. The data of Chhabra et al. (4) delineating the structural requirements for analogues of OdDHL to mediate suppression of lymphocyte proliferation indicated that the acyl chain length, the lipophilicity of the molecule, and the presence of the homoserine lactone ring were all important components of the required structure. This suggested the possibility that OdDHL could insert within the lipid bilayer and act by disrupting the association of molecules, including the TCR, within the plasma membrane. To investigate this possibility, TCR-transgenic CD4 T cells were stimulated either with APCs plus peptide antigen, to model a receptor-mediated stimulation, or with Ca^{2+} ionophore (ionomycin) and PMA, to model stimulation in which plasma membrane-dependent receptor interactions were bypassed. Cells were incubated either with 0.05% DMSO (control) or with 1 or 5 μM OdDHL in 0.05% DMSO for the duration of the cultures. As previously, culture supernatants were harvested at 48 h and assayed by ELISA for IFN-γ. IL-4 was not assayed in this experiment, since the ionomycin-PMA stimulation generates negligible levels of IL-4 in this model. The results of this experiment are shown in Table 2 and indicate that while ionomycin and PMA did not induce as much IFN-γ production as antigen, the production was clearly inhibited by OdDHL and may even be more sensitive: the inhibition was greater at 1 μM OdDHL for ionomycin-PMA stimulation than for antigen stimulation, although this difference did not reach statistical significance ($P = 0.11$). Ca^{2+} ionophore directly up-regulates intracellular free calcium concentrations, while PMA can activate protein kinase C directly: the combination of these can activate downstream signaling pathways from TCR and other cell surface receptors. Since this mode of

TABLE 1. Quorum-sensing activity of OdDHL correlates with immunomodulatory activity

OdDHL	Starting concn of OdDHL (μM)	Relative QS activity at[a]:		% Control IFN-γ production[b]	% Control IL-4 production[b]
		90 min	Time of T-cell addition		
Fresh[c]	10	ND	10 ± 2.3	1.0 ± 0.2	6.7 ± 4.3
	4	ND	4 ± 0.5	40.0 ± 17.0	47.6 ± 14.8
	1	ND	1 ± 0.2	83.9 ± 26.4	98.8 ± 13.7
Preincubated[d]	10	7.7	5 ± 1.5	40.8 ± 24.5	35.8 ± 24.4
	4	ND	2.8 ± 0.3	64.5 ± 18.9	109 ± 6.7
	1	0.9	Not detectable	92.0 ± 8.6	116 ± 13
Residual[e]	10	ND	0.6 ± 0.5	82.0 ± 20.1	86.3 ± 23.7
	4	ND	Not detectable	98.4 ± 9.0	94.2 ± 34.9
	1	ND	Not detectable	104.2 ± 6.2	102 ± 18.6

[a] Quorum sensing (QS) activity of OdDHL compared to known concentration of freshly prepared OdDHL as assayed in *A. tumefaciens* bioassay. ND, not done.
[b] Cytokine production as a percentage of that observed in DMSO control cultures (90-minute timepoint; two replicates). All other data are expressed as mean ± standard deviation for eight replicates.
[c] OdDHL added to wells for 4 h at 37°C and then removed before addition of cells and antigen.
[d] OdDHL added to wells and incubated at 37°C before addition of cells and antigen.
[e] OdDHL prepared and added to cells at time of antigen stimulation.

TABLE 2. Comparison of the effect of OdDHL on IFN-γ production induced in T cells by antigen (MCC_{T102S}) or ionomycin + PMA stimulation[a]

Stimulus	Control (0.05% DMSO)	1 μM OdDHL in 0.05% DMSO	% Inhibition by 1 μM OdDHL	5 μM OdDHL in 0.05% DMSO	% Inhibition by 5 μM OdDHL
Antigen (MCC_{T102S}), 5 μg/ml	70.1 ± 12.0	54.8 ± 14.3 ($P = 0.44$)	21.8	27.2 ± 9.3 ($P < 0.03$)	61.2
Ionomycin (1 mg/ml) + PMA (8 ng/ml)	5.54 ± 1.31	2.75 ± 0.84 ($P = 0.11$)	50.4	1.53 ± 0.20 ($P < 0.02$)	76.4

[a] Results are expressed as mean nanograms of IFN-γ/ml ± standard error of the mean ($n = 5$). Statistical differences between control and OdDHL-treated cells were assessed using an unpaired t test.

stimulation was also inhibited by OdDHL, it is likely that OdDHL acts at an intracellular site, possibly one common to several receptors. This reduces the likelihood, but does not exclude the possibility, that it also affects membrane fluidity or the aggregation of the TCR.

OdDHL does not induce death in T lymphocytes. To determine whether OdDHL inhibits T-cell function by inducing cell death, either apoptosis or necrosis, spleen cells were resuspended in CCM and treated with a range of concentrations of OdDHL (1 to 10 μM) in 0.05% DMSO or with 0.05% DMSO alone for 2 h at 37°C, washed once, and labeled with FITC-conjugated CD3 (total T cells), CD4 (Th cells and low levels on monocytes), and B220 (B cells) monoclonal antibodies, together with propidium iodide, and analyzed by flow cytometry. The results of this experiment are shown in Table 4. This demonstrates that OdDHL at 4 to 10 μM does cause cell death in nonlymphoid spleen cell populations (including monocytes and neutrophils) and in B cells. Detectable cell death above control in CD4 T cells (8.8%) was observed only at 10 μM OdDHL, at which concentration cytokine production is almost completely inhibited. Its effect on the survival of minority populations, such as dendritic cell APCs, has not been confirmed. Thus, while OdDHL clearly has direct effects on anti-CD3 (Fig. 2A)- or ionomycin-PMA (Table 2)-stimulated T cells, it is possible that a component of inhibition of antigen-specific stimulated T-cell cytokine production is due to effects of OdDHL on APCs.

Effects of OdDHL on primary and secondary $CD4^+$-T-cell responses. Previous experiments in nontransgenic mice on the effects of OdDHL on antigen-specific stimulation of T cells have of necessity evaluated only secondary responses, as in vivo immunization of mice is required to generate a detectable in vitro response. Our data (14) suggested that OdDHL treatment at the time of in vivo immunization caused increases in cytokine production in in vitro secondary responses initiated 8 days later in the absence of OdDHL. In the only other report of the effects of OdDHL on TCR-transgenic T-cell responses to antigen, Smith et al. reported that, after exposure to OdDHL in a primary response, CD4 T cells showed increased production of IFN-γ when restimulated in the absence of OdDHL (17). No analysis of the effects of OdDHL present during secondary responses has been performed. Since in chronic *P. aeruginosa* lung infections, ongoing T-cell and B-cell immune responses are clearly detectable, it is important to characterize whether these secondary responses are in fact resistant to OdDHL actions or are as susceptible to inhibition as primary responses. Our TCR-transgenic mouse model allows us to compare the effects of OdDHL on primary and secondary CD4 T-cell-mediated cytokine production in vitro. Spleen cells from TCR-transgenic mice were stimulated with 10 μM MCC_{T102S} in the presence of 5 μM OdDHL in 0.05% DMSO or 0.05% DMSO alone. Supernatants were harvested from some replicates after 2 days and assayed by ELISA for IL-4 and IFN-γ production. After 3 days, the cultures were split into two equal parts, and fresh 5 μM OdDHL in fresh culture medium, or fresh culture medium alone, was added to cultures. On day 6, cells were harvested, washed, counted, and adjusted to a constant viable-cell concentration before being restimulated with fresh irradiated B10.BR splenic APCs plus MCC_{T102S} peptide in the presence or absence of 5 μM OdDHL. Cytokine production was assayed by ELISA 48 h after secondary stimulation. The results of this experiment are shown in Table 3. This clearly demonstrates that OdDHL inhibits both IL-4 and IFN-γ production by T cells in both primary and secondary stimulations. Additionally, it shows that secondary stimulation in the absence of OdDHL does not permit recovery of cytokine production by cells initially stimulated in the presence of OdDHL.

To address the question of whether the presence of OdDHL during primary stimulation could alter the Th1-Th2 balance of a subsequent secondary response, a range of concentrations of antigen peptide was used to stimulate CD4 T cells in primary

TABLE 3. Comparison of sensitivities of primary and secondary $CD4^+$ T-cell responses to OdDHL[a]

Treatment primary response	IFN-γ primary response (ng/ml)	IL-4 primary response (pg/ml)	Treatment secondary response	IFN-γ secondary response (ng/ml)	IL-4 secondary response (pg/ml)
0.05% DMSO	53.9 ± 4.6	32.9 ± 3.7	0.05% DMSO	83.9 ± 34.7	168 ± 47
			5 μM OdDHL	30.9 ± 12.0 (36.8%)[c]	70.6 ± 23.7 (42.0%)[c]
5 μM OdDHL	12.5 ± 3.8 (23.2%)[b]	10.7 ± 0.7 (32.5%)[b]	0.05% DMSO	1.55 ± 0.76	11.1 ± 2.7
			5 μM OdDHL	0.49 ± 0.30 (31.6%)[d]	ND[e]

[a] All results are expressed as mean ± standard error of the mean of data from five individual mice.
[b] Percent DMSO control.
[c] Percent DMSO-DMSO control.
[d] Percent OdDHL-DMSO control.
[e] ND, not detectable (limit of detection, 6 pg/ml).

TABLE 4. Effects of OdDHL on viability of splenic leukocyte populations

Parameter	Value			
	$CD3^+$	$CD4^+$	$B220^+$	$CD3^-$ $B220^-$
% Total spleen cells[a]	20.5 ± 3.7	21.3 ± 3.6	51.0 ± 3.3	29.5 ± 3.5
% Death of each population in[b]:				
CCM	4.7	7.7	12.6	14.7
DMSO	7.6	6.6	9.3	11.9
1 μM OdDHL	6.3	7.1	5.4	11.7
4 μM OdDHL	8.5	7.7	9.8	18.9
10 μM OdDHL	11.0	15.4	37.4	47.8

[a] Average ± standard deviation for six determinations.
[b] Results are expressed as percent cell death within each population, taking each individual population as 100%. The values are average of two individual experiments.

culture, followed by secondary stimulation with a fixed concentration of peptide and analysis of cytokine production. The way in which the cytokine response of the TCR-transgenic CD4 T cells in our model varied with antigen concentration and affinity has been described by others (5, 15), and low concentrations of antigen during primary stimulation have been reported to favor the generation of Th2 cells detected in secondary stimulation (15). Transgenic MACS-purified CD4 T cells were stimulated with irradiated B10.BR spleen cells pulsed with concentrations of antigen peptide ranging from 0.01 to 10 μM in the presence or absence of 5 μM OdDHL. Cultures were split 1:3 on day 3, and since OdDHL had been demonstrated to produce its effects within the first 2 h after stimulation, fresh culture medium without OdDHL was added. As before, cells were harvested on day 6, counted, resuspended at a fixed concentration, and restimulated with a constant concentration of 5 μM peptide in the presence or absence of 5 μM OdDHL. Cytokine production at 48 h of secondary culture was evaluated by ELISA (Fig. 3). Interestingly, the presence of OdDHL during the first 3 days of primary culture leads to only small reductions in the overall number of viable cells recovered on day 6 (data not shown), consistent with the observation that OdDHL causes minimal cell death in CD4 T cells. However, this is not reflected in the level of cytokine production by a constant number of cells after secondary restimulation. The results of this experiment are shown in Fig. 3, which shows that cells stimulated with low concentrations (<0.1 μM) of antigen in primary stimulation do in fact produce more IL-4 in secondary stimulation than do cells stimulated with high (10 μM) concentrations of antigen, but the level of inhibition by OdDHL remained essentially constant over the antigen concentration range tested. Thus, it appears that rather than favoring the emergence of a Th1- or Th2-biased population, OdDHL inhibits the development of all effector CD4 T cells. However, it is of interest to note that in the secondary stimulation, IFN-γ production appears to be more sensitive to OdDHL inhibition than does IL-4 (Fig. 3).

The role of antigen affinity in the action of OdDHL. Previous reports from other laboratories have suggested that OdDHL could be proinflammatory (17) or favor the development of a Th2 response (20), while our data suggested that OdDHL inhibited the development of all CD4 T cells rather than favoring one subset or another (14). Since these studies used different model antigens, we considered the possibility that the affinity of the TCR-antigen interaction, which affects the intensity of downstream signaling events, could modulate the inhibitory effects of OdDHL on that process. The affinity of the TCR-antigen interaction has been demonstrated in several models to have an influence on the outcome of a CD4 T-cell response, with high-affinity antigens leading to more Th1-type responses and lower-affinity antigens tending to produce Th2-biased responses. Thus, we analyzed the effects of stimulation with two different peptides, the wild-type MCC peptide and the altered lower-affinity MCC_{T102S} peptide, on the inhibition by OdDHL of cytokine production in TCR-transgenic CD4 T cells. Splenocytes from TCR-transgenic mice were stimulated with a range of concentrations of both wild-type peptide and MCC_{T102S} peptide in the presence or absence of 5 μM OdDHL, and cytokine production was assessed at 48 h. The results of this experiment are shown in Fig. 4. It can be seen that, as for the secondary responses described above, the degree of inhibition of each cytokine in these primary responses

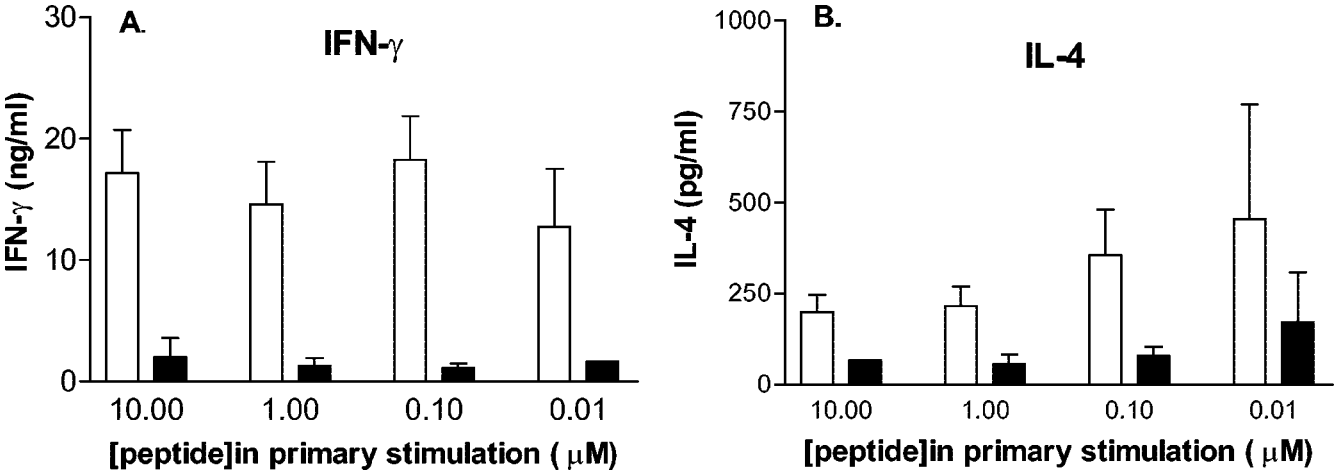

FIG. 3. Cytokine production during secondary stimulation of CD4 T cells after primary stimulation with a range of antigen concentrations in the presence or absence of OdDHL. Shown are cells treated with 0.05% DMSO alone (white bars) and cells treated with 5 μm OdDHL in 0.05% DMSO (black bars). The results are expressed as the mean plus standard error of the mean of two or three individual experiments.

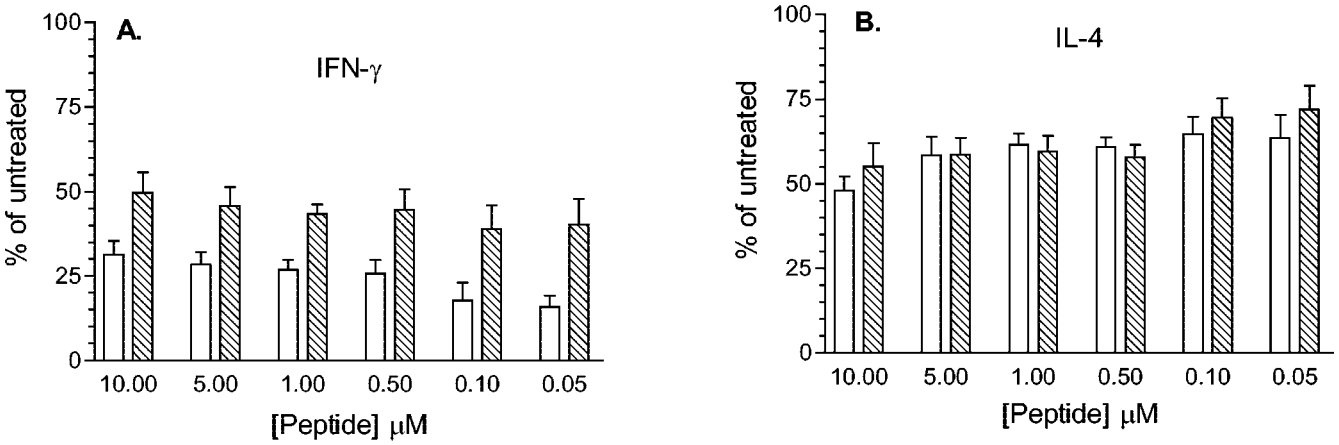

FIG. 4. Effects of varying antigen affinity on the inhibition of cytokine production in primary stimulation by OdDHL. The results are expressed as the percentage of control (DMSO-treated) IFN-γ or IL-4 production stimulated by wild-type MCC peptide (hatched bars) or by MCC_{T102S} (white bars) at each concentration and are the mean plus standard error of the mean of at least three experiments.

varied little over the range of antigen concentrations tested. However, and most strikingly, while OdDHL inhibited IL-4 production by 30 to 50% for both antigens, IFN-γ production was much more strongly inhibited when CD4 T cells were stimulated with altered MCC_{T102S} peptide than with wild-type peptide. Thus, it is clear that the precise outcome of the inhibition of T-cell function by OdDHL can be modulated by the affinity of the antigen to which the T cells are responding.

DISCUSSION

The data presented in this paper strongly suggest that the effects of OdDHL on T-cell responses are an amalgam of several different processes. First, it is clear that for both anti-CD3 and antigen-specific stimulation of CD4 T cells, OdDHL inhibits a crucial step within the CD4 T cells that is complete within the first 2 h after initiation of stimulation, making the most likely target some component or components of important signal transduction pathways involved in T-cell activation. The target of this inhibition remains uncertain, but the data showing inhibition by OdDHL of ionomycin-PMA stimulation makes it unlikely that its inhibitory effects are solely due to insertion of OdDHL into the plasma membrane and a consequent inhibition of TCR aggregation and immune synapse formation. This is also supported by the observation of Williams et al. (21) that OdDHL can enter COS cells and initiate activation and nuclear translocation of a transfected LasR construct. Secondly, while it is evident that OdDHL has direct effects on CD4 T cells, it does not, at the concentrations used, cause significant death of these cells, although it does appear to cause the death of some B cells and of nonlymphoid splenic leukocytes (Table 4). Thus, it is possible that a component of its effects on antigen-specific T-cell stimulation may be due to toxic effects on APCs. Thirdly, the analysis of the effects of OdDHL on primary and secondary stimulation of CD4 T cells suggests strongly that rather than selectively inhibiting the development of either Th1 or Th2 cells, OdDHL inhibits the development of all effector CD4 T cells. However, a possible explanation for the reports of preferential effects of OdDHL on either Th1 (17) or Th2 (20) responses can be deduced from two pieces of data. First, the observations that in secondary stimulation, IFN-γ production appears to be more sensitive to OdDHL than does IL-4 means that in secondary responses, a preferential development of Th2 responses could occur, and OdDHL treatment could favor a Th2-like bias for a long-term response. Secondly, the fact that the affinity of the antigen-TCR interaction modulates the outcome of OdDHL inhibition means that the overall effect of OdDHL on a response could depend on the affinity of the antigen in question for the relevant TCR.

From the data on anti-CD3 stimulation of T cells, it is clear that OdDHL has direct effects on T cells and inhibits a process that is crucial in the initial stages of T-cell activation. However, it is also clear from the data in Table 3, and from the fact that OdDHL addition at times later than 2 h after activation has no effect on subsequent cytokine production, that OdDHL does not significantly affect the overall viability of CD4 T cells. By contrast, OdDHL does appear to induce cell death in some non-CD4-positive splenic leukocytes, consistent with the observations of Tateda et al. (19), who observed induction of apoptosis and necrosis in human neutrophils and monocytes in the presence of OdDHL. The observation that OdDHL appears to induce the death of some B cells is a novel finding that we are following up. Interestingly, the B-cell population affected has lower than average expression of B220, a characteristic of B-1 B cells in the mouse (1).

The observation that the affinity of the antigenic peptide major histocompatibility complex (MHC) for the T-cell receptor also appears to modulate the effects of OdDHL on T-cell cytokine production is also interesting and strengthens the speculation that OdDHL acts on a signal transduction pathway whose activity is dependent on the intensity of the signal resulting from TCR ligation, since otherwise, no difference would be expected in the effects for different antigens. This effect of antigen affinity could, of course, be a consequence of the effects of OdDHL on either APCs or T cells, a distinction that can be made only when the effects of OdDHL on APCs are clarified. The two peptide antigens used in this study have been reported to have essentially equivalent affinities for the

MHC molecule that presents them to the TCR-transgenic CD4 T cells (15), so the differences in the effects of OdDHL on the two responses are less likely to be due to differences in the amount of antigen presented to T cells than to differences in the affinity of the MHC-antigen-TCR interaction and the consequent activating signal strength.

The work outlined in this report indicates strongly that the effects of OdDHL in even such a well-defined system as antigen-specific stimulation of purified CD4 T cells can be modulated by a range of factors, including antigen affinity and concentration. It also provides further evidence that OdDHL is acting at an intracellular site and shows that relatively early stages of T-cell activation are crucially affected. Our data virtually eliminate the possibility that the highly polar and lipophilic OdDHL molecule exerts its effects solely by inserting into the plasma membrane and thereby altering membrane fluidity and receptor aggregation, although this cannot be excluded as a minor component of its mode of action. It is also unlikely that, in T cells at least, it is acting at a posttranscriptional level, since (i) we have previously demonstrated reduced expression of cytokine mRNA in OdDHL-treated T cells (14) and (ii) OdDHL is required to be present during the first 2 h after antigen or mitogen stimulation of T cells. These data combined strongly suggest that OdDHL modulates some aspect of the TCR-dependent or costimulatory signal transduction pathways. Smith et al. observed that in respiratory epithelial cells, OdDHL treatment led to increased activation of NF-κB (16) and COX-2 (18) but were unable to identify where in the pathway leading to this activation OdDHL bound. Activation of NF-κB in T cells is known to lead to increased activation and cytokine production (9), so it is unlikely to be the mechanism operative in the inhibition by OdDHL of proliferation (20) and cytokine production (14, 20) by T lymphocytes. So, while we have narrowed down the possible modes of action of OdDHL in T cells, its precise binding target in mammalian cells remains to be identified. Clarification of this binding target or targets awaits proteomic or other global analysis of the effects of OdDHL in T cells.

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