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CD1-Restricted Recognition of Exogenous and Self-Lipid Antigens by Duodenal $\gamma\delta^+$ T Lymphocytes[1]

Anna M. Russano,* Gabrio Bassotti,† Elisabetta Agea,* Onelia Bistoni,* Alessandro Mazzocchi,† Antonio Morelli,† Steven A. Porcelli,‡ and Fabrizio Spinozzi[2]*

$\gamma\delta$ T cells are present in the mucosal intestinal epithelia and secrete factors necessary to maintain tissue integrity. Ags recognized by these cells are poorly defined, although in mice non-classical MHC class I molecules have been implicated. Since MHC class I-like CD1 receptors are widely expressed at the surface of epithelial and dendritic intestinal cells and have the capacity to present lipid Ags to T cells, we hypothesized that these molecules might present autologous and/or exogenous phospholipids to intestinal $\gamma\delta$ T lymphocytes. Intraepithelial T lymphocytes from normal human duodenal mucosal biopsies were cloned and exposed to natural and synthetic phospholipids using CD1a-, CD1b-, CD1c- or CD1d-transfected C1R lymphoblastoid or HeLa cell lines as APCs. Their cytolytic properties and regulatory cytokine secretion were also examined. Most clones obtained from duodenal mucosa (up to 70%) were TCR$\alpha\beta^+$, and either CD4$^+$ or CD8$^+$, whereas 20% were CD4$^-$CD8$^-$ (6 clones) or TCR$\gamma\delta^+$ (12 clones). A relevant percentage (up to 66%) of TCR$\gamma\delta^+$ but few (<5%) TCR$\alpha\beta^+$ T cell clones responded to synthetic and/or natural phospholipids presented by CD1 molecules, as measured by both [^{3}H]thymidine incorporation and IL-4 release assays. A Th1-like cytolytic and functional activity along with the ability to secrete regulatory cytokines was observed in most phospholipid-specific $\gamma\delta$ T cell clones. Thus, a substantial percentage of TCR$\gamma\delta^+$ but few TCR$\alpha\beta^+$ from human duodenal mucosa recognize exogenous phospholipids in a CD1-restricted fashion. This adaptive response could contribute to mucosal homeostasis, but could also favor the emergence of inflammatory or allergic intestinal diseases. *The Journal of Immunology,* **2007, 178: 3620–3626.**

Processing and presentation of foreign Ags at mucosal surfaces, as well as induction and clonal expansion of mucosal Ag-specific effector T lymphocytes, occurs at specific sites that are marked by the presence of specialized epithelium with Ag uptake and transporting capacity and organized lymphoid tissue (1). In humans, the oral and nasal pharynx is monitored by a ring of lymphoid tissue, and lymphoid follicles are present in the bronchi. Single lymphoid follicles are also distributed along the intestine and increase in frequency in the distal ileum and colon, where the microbial flora is abundant and diversified (2). Gut epithelial lymphocytes are mainly represented by T cells, often displaying cytolytic activity, and usually defined intraepithelial lymphocytes (IELs)[3] (3). A growing bulk of evidence suggests that IELs are potent, rapidly activated cytolytic and immunoregulatory effectors that can protect their host tissues from infection, cell transformation, and uncontrolled infiltration by systemic cells.

The regulatory properties of IELs are suggested primarily by the fact that in the human small bowel 10–20 IELs are present per 100 villous enterocytes. Therefore, given the enormous surface area of such epithelia, resident IELs may comprise a substantial fraction of total T cell numbers of a given subject (4). Despite the tremendous load of foreign Ag ingested daily and the enormous number of lymphocytes resident in the mucosa, the normal state of the gut is characterized by immune hyporesponsiveness such that immune responses generally occur only when real danger signals are present (3).

Approximately 50% of human IELs are TCR$\alpha\beta^+$ T lymphocytes that express the CD8$\alpha\beta$ heterodimer form of the CD8 coreceptor (hereafter referred to as CD8$^+$TCR$\alpha\beta^+$ T cells). The remainder is constituted by several other unconventional (i.e., non MHC-restricted) T cell types, like TCR$\gamma\delta^+$ (either double negative (DN) for both CD4 and CD8, or positive for the homodimer CD8$\alpha\alpha$) and TCR$\alpha\beta^+$CD8$\alpha\alpha^+$ T lymphocytes (3). CD8$^+$TCR$\alpha\beta^+$ T lymphocytes are primarily cytolytic and also secrete Th1-type cytokines (5); are predominant in the large bowel, which harbors the greatest microbial Ag load; and comprise alloreactive T cells that contribute to graft-versus-host disease (6). In contrast, unconventional TCR$\gamma\delta^+$ and TCR$\alpha\beta^+$CD8$\alpha\alpha^+$ cells, while sharing cytolytic properties with TCR$\alpha\beta^+$ cells, seem to recognize self-molecules induced by infection or cell transformation in the mouse model (7). Neither the identity nor the nature (proteinaceous or nonproteinaceous) of the putative autoantigen(s) is presently known, although non-classical MHC Ags have been strongly implicated.

Because CD1 molecules, a family of well-described MHC class I-like lipid Ag-presenting molecules, are widely expressed at the surface of both epithelial (8) and dendritic cells in the intestine (9), we tested the hypothesis that these may be involved in recognition of autologous or exogenous natural phospholipids (PLs) by conventional TCR$\alpha\beta^+$ and/or TCR$\gamma\delta^+$ T cells in the gut. Our results

*Laboratory of Experimental Immunology and Allergy, Section of Internal Medicine and Oncological Sciences, †Gastrointestinal and Hepatology Section, Department of Clinical and Experimental Medicine, University of Perugia Medical School, Perugia, Italy; and ‡Department of Microbiology and Immunology, Albert Einstein College of Medicine, New York 10461

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[2] Address correspondence and reprint requests to Prof. Fabrizio Spinozzi, Experimental Immunology and Allergy, Department of Clinical and Experimental Medicine, University of Perugia, Policlinico Monteluce, I-06122 Perugia, Italy. E-mail address: spinozzi@unipg.it

[3] Abbreviations used in this paper: IEL, intraepithelial lymphocyte; DN, double negative; PC, L-α-phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, phospholipid; iNKT, invariant NKT.

strongly implicate duodenal mucosal TCR$\gamma\delta^+$ T cells in such recognition, and suggest that these may potentially exert a regulatory role in maintaining homeostasis in the gut by preventing unwanted immune responses toward ingested food Ags or autologous damaged and/or apoptotic epithelial cells.

Materials and Methods

Duodenal mucosal biopsies

Multiple duodenal samples were obtained from 10 subjects undergoing upper endoscopy for gastric cancer screening, after a careful explanation of the aims of the study and after obtaining written informed consent. No patient complained of gastrointestinal symptoms. A standard upper endoscopy procedure was conducted by means of Olympus 145L standard videoendoscope to exclude the presence of organic diseases; therefore, three to five bioptic samples were obtained from the normal-appearing duodenal mucosa of each subject with completely normal endoscopic findings. All endoscopic procedures were conducted by one of the authors (G.B.).

Isolation of IELs and cloning procedures

For IEL isolation a previously described procedure, which avoids the use of enzymes or chelating substances (EDTA) affecting lymphocyte function, was adopted (10). Briefly, after sampling, two duodenal biopsies were placed in a 50-ml screw-cap tube containing 45 ml of PBS with 2 mM DTT (Invitrogen Life Technologies) and immediately transported to the analysis laboratory. After vortexing to eliminate epithelium, cell suspensions were centrifuged and resuspended in RPMI 1640/5% FBS containing 10 mM HEPES buffer, 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen Life Technologies). Purification of IELs included pipetting of the cell suspensions to release lymphocytes from clumps of enterocytes, passing the cell suspension through two layers of cheesecloth (Fischer Scientific) to remove mucus and sheets of epithelium, and centrifugation over 30% (v/v) Percoll density gradient (Pharmacia). The cells that passed through 30% Percoll were resuspended in 45% Percoll. A discontinuous Percoll gradient was then made by layering the volume of 45% Percoll above 15 ml of 75% (v/v) Percoll in a 50-ml tube. The gradient was centrifuged at 500 rpm for 30 min at room temperature and the cells recovered from the 45 to 75 interface were washed three times in PBS. The viability and purity of IELs suspensions were assayed by trypan blue and staining with FITC-conjugated anti-CD3 mAb (Serotec).

For cloning procedures, we adopted the limiting dilution technique with heterologous irradiated mononuclear cells as feeder and PHA-P as polyclonal stimulus. Briefly, samples were seeded at 0.5, 1, and 2 cells/well in 96-well round-bottom plates (Nunclone; Nunc) with 10^5 irradiated (5000 rad) feeder cells, 1% (v/v) PHA-P, and rIL-2 (20 U/ml) in complete medium supplemented with 10% heat-inactivated human AB serum. Growing microcultures were then expanded at weekly intervals with 10^5 irradiated feeder cells and rIL-2.

Antibodies

The phenotype of clones was assayed with the following mAbs: FITC-conjugated anti-TCR$\alpha\beta$ (clone BMA031), anti-TCR Vα24 (clone C15), anti-TCR$\gamma\delta$ (clone 5A6.E9), anti-Vδ1 (clone TS8.2), and anti-CD4 (clone RPA-T4) (all from Serotec); purified anti-CD8β (clone 2ST8.5H7, IgG2a); PE-conjugated anti-CD8α (clone LT8; Serotec), and anti-CD161 (clone DX12: BD Biosciences). For blocking experiments, we adopted the following purified mAbs: anti-CD1a (clone NA1/34-HLK; IgG2a), anti-CD1b (clone M-T101; IgG1), anti-CD1c (clone L161; IgG1), anti-CD1d (CD1d42; IgG1) (all from BD Biosciences), W6/32 (anti-MHC class I; IgG2a, American Type Culture Collection), L243 (anti-HLA DR; IgG1; American Type Culture Collection), and N-S.4.1 (IgM control; American Type Culture Collection). In all blocking experiments, mAbs were added as ascites (1/200 final dilution) or as purified mAbs (20 μg/ml).

Immunofluorescence analysis

For immunofluorescence analysis, cells (1×10^6/ml) were incubated with FITC- or PE-conjugated mouse anti-human mAbs on ice for 30 min, washed, and analyzed by flow cytometry (FACScan; BD Biosciences). Control Abs for immunophenotype experiments were FITC- or PE-conjugated polyclonal $F(ab')_2$ goat anti-mouse IgG (Serotec). Results were expressed as percentage of positive cells compared with staining with isotype-matched control mAbs.

CD1$^+$ APCs

Monocyte-derived dendritic cells were generated from human blood monocytes of normal volunteers and induced to differentiate and express CD1a, CD1b, CD1c, and CD1d by incubation with GM-CSF and IL-4 as previously described (11). The lymphoblastoid cell lines C1R were transfected with the expression vector pSRα-Neo into which cDNAs encoding either CD1a, CD1b, CD1c, and CD1d were inserted (12). HeLa cells were either mock transfected (HeLa mock) or CD1a, CD1b, CD1c, or CD1d transfected (13).

Lipid Ags

The following synthetic reagents were purchased from Sigma-Aldrich and used without further purification: 16:0/16:0 L-α-dipalmitoyl phosphatidylcholine (PC) and 18:1/18:1 dioleoyl phosphatidylethanolamine (PE). Synthetic 16:0/18:2 PE and 16:0/18:2 phosphatidylglycerol (PG) were purchased from Avanti Polar Lipids. Natural PLs were extracted from cypress pollen according to the method of Folch. The methanol/chloroform extract was evaporated nearly to dryness under N_2 and chromatographed on silica gel G TLC plates using chloroform/methanol/water (65:25:4, by volume) as the solvent. PL classes were separated by two-dimensional TLC (6.5 $\times$ 6.5 cm; PE SIL G 250 μm; Whatman) with 1) chloroform/methanol/1.6 M ammonia (70:30:5, by volume) and 2) chloroform/methanol/acetone/acetic acid/water (75:15:30:15:7.5, by volume). Spots were revealed by staining with dichlorofluorescein, scraped off the plate, and eluted with chloroform/methanol/acetic acid/water (50:39:1:10, by volume). Samples were freed from dichlorofluorescein by washing three times with 3 ml of 4 N ammonia and three times with methanol/water (45:10, by volume). Trace amounts of silica gel were removed by centrifugation. Finally, PLs were quantified by phosphorus assay after digestion with 70% perchloric acid (14). Naturally occurring PC and PE from egg yolk, liver, soy, or brain tissue were obtained from Avanti Polar Lipids. All PL suspensions were prepared in absolute ethanol. All dried reagents were resuspended in absolute ethanol and stored at -20°C. In proliferation and cytokine assays, lipid Ags were added to cultured cells at a concentration ranging from 2 to 10 μg/ml.

Proliferation assays

In brief, 5×10^4 T cells were plated in triplicate in 96-well flat-bottom plates (Falcon; BD Biosciences) with either 5×10^4 irradiated (5000 rad) C1R transfectants, CD1$^+$ dendritic cells, or mitomycin C-treated HeLa mock and HeLa CD1c cells as APCs. In the mAb blocking experiments, CD1$^+$ dendritic cells were preincubated with anti-CD1a, -b, -c, and -d mAbs for 6 h before culturing with lipid Ag and T cells. After 5 days of culture, all plates were pulsed with 1 μCi of [^{3}H]thymidine and harvested 16 h later using a Tomtec harvester. The filter papers were counted on a Beckman Beta Counter (Beckman Coulter) and results are expressed as cpm $\pm$ SEM.

Cytolytic assays

Cytolytic activity was measured by a 4-h chromium release assay. The targets used were C1R lymphoblastoid cells either mock transfected (C1R Mock), CD1b transfected (C1R CD1b), or CD1d transfected (C1R CD1d). For blocking experiments, the targets were incubated with 20 μg/ml relative mAbs for 30 min at room temperature before adding effector cells. All assays were performed in triplicate, and results are expressed as percent specific ^{51}Cr release $\pm$ SEM.

Cytokine production

In brief, 5×10^5 T cells from either $\gamma\delta$ or $\alpha\beta$ T cell clones were cultured with 5×10^5 C1R transfectants with or without lipid Ags. PHA-P (1/4000) was added as positive control. Supernatants were harvested after 2 days of culture. Cytokine release was determined for IL-4, IL-10, TGF-β, and IFN-γ by commercial ELISA kits (Quantikine; R&D Systems). Results are expressed as picograms per milliliter $\pm$ SEM.

Results

Isolation and phenotypic analysis of duodenal T cell clones

The percentages of CD3$^+$ lymphocytes among total mononuclear cells extracted from duodenal mucosal specimens ranged from 4 to 10%, but after enrichment by Percoll density gradient increased to 81 to 92%. Following this procedure, T cells from each donor were further expanded in complete medium for 4 days and, subsequently, cloned at limiting dilution after culturing with PHA-P in

Table I. *Characteristics of lipid-reactive IEL T cell clones from duodenal mucosa*[a]

Clone	Phenotype (TCR$\gamma\delta^+$)	Autologous APC[b]	Reactivity				
			CD1 transfectants[c]	Synthetic PLs	Natural PLs	IL-4	IFN-γ
D3/1.8	$CD4^+V\delta1^+$	No	C1Rd	18:2/18:2 PE	Pollen and soy PE, ++	++	++
D3/7.8	$CD4^-CD8^-V\delta1^+$	Yes	C1Rd	16:0/16:0 PC	Egg, ++; liver, ++	+	+++
D5/5.10	$CD4^+V\delta1^+$	No	C1Rc	16:0/16:0 PC	Egg, ++; liver, +	+	++
D5/9.7	$CD4^-CD8^-V\delta1^+$	No	C1Rc	18:2/18:2 PE	Brain and liver PE, ++	++	++
D7/1.9	$CD4^+V\delta1^+$	No	C1Ra/d	18:2/18:2 PE	Pollen and soy PE, ++	++	++
D7/5.11	$CD4^+V\delta1^+$	No	C1Ra	16:0/16:0 PC	Egg, ++	+	+++
D9/4.14	$CD4^-CD8^-V\delta1^+$	Yes	C1Rc	16:0/16:0 PC	nd[d]	+	+++
D10/9.3	$CD4^-CD8^-V\delta1^+$	Yes	C1Ra	16:0/18:2 PG	Brain, ++; liver, ++	+	+++
Clone	(TCR$\alpha\beta^+$)						
D5/9.5	$CD8^+\alpha\beta^+$	No	C1Ra	16:0/18:2 PE	nd	+++	+
D6/1.3	$CD4^+\alpha\beta^+$	No	C1Rc	18:2/18:2 PE	Brain, ++; liver, +	++	++
D7/5.15	$CD4^+\alpha\beta^+$	No	C1Ra	16:0/16:0 PC	Egg, ++	+	+++

[a] Ag reactivity was measured by IL-4 production in 48-h culture supernatants: between 10 and 50 pg/ml (0), between 60 and 150 pg/ml (+), between 160 and 250 pg/ml (++), <260 pg/ml (+++/++++).
[b] Reactivity to autologous APC as revealed in autologous MLR without adding exogenous PLs.
[c] Reactivity to CD1 transfectants refer to the restriction of the response to exogenous added PLs.
[d] nd, None detected.

the presence of 50 U/ml exogenous IL-2. PHA-stimulated duodenal IEL give rise to various T cell clones with diverse phenotypes. From a total number of 320 T cell clones obtained from duodenal mucosa of the subjects studied, we selected 90 clones that showed the most robust growth in vitro and proliferation rates for subsequent functional studies. Most clones (up to 70%) expressed a conventional TCR$\alpha\beta^+$ and were either $CD4^+$ or $CD8^+$, whereas 20% of them were $CD4^-CD8^-$ TCR$\alpha\beta^+$ (6 clones) or TCR$\gamma\delta^+$ (12 clones).

CD1-restricted recognition of PLs by duodenal T cell clones

Although the cell lines were expanded without adding exogenous Ags, a relevant percentage of PHA-derived TcR$\gamma\delta^+$ T cell clones (8 of 12) but few TCR$\alpha\beta^+$ T cell clones (3 of 78) were found to be responsive to in vitro stimulation with synthetic and/or natural PL Ags by means of both [^{3}H]thymidine incorporation and IL-4 release assays (Table I). The type of Ag-presenting molecules involved in the recognition of PLs was investigated with the use of lymphoblastoid C1R and HeLa cell lines (mock or CD1 transfected) as APCs. All but one clone showed restriction of their responses to PLs by a single CD1 isoform (clone D7/1.9; Table I), whereas there was no response with mock-transfected C1R or HeLa cells (data not shown). The specificity of such recognition was also confirmed by experiments performed with autologous dendritic cells expressing detectable surface CD1 molecules exposed to blocking specific mAbs. Pretreatment with anti-CD1a, CD1c, and CD1d mAbs inhibited proliferation of most PL-specific T cell clones in a manner congruent with the results obtained using transfected APCs (data not shown). Anti-CD1b or anti-MHC mAbs did not modify the responses to synthetic PLs.

An example of such functional studies performed on all tested clones is illustrated in Fig. 1*A*: the $CD4^+$TCR$V\delta1^+$ clone D5/5.10 reacted with synthetic (16:0/16:0) dipalmitoyl PC, almost exclusively in a CD1c-restricted manner. The specificity of CD1 restriction was demonstrated by either mAb blocking experiments (Fig. 1*B*) or by using C1R transfectants expressing CD1c molecules (Fig. 1*C*) as APCs. It should be noted that the D5/5.10 T cell clone proliferated markedly also to HeLa-CD1c pulsed with lipid Ag, but not to HeLa mock cells. Thus, it can be assumed that D5/5.10 T cells did not recognize MHC class I-related proteins such as MHC class I chain-related gene A product, since these cells did not react with the HeLa mock cell line that expresses MHC class I chain-related gene A product (15).

To demonstrate that TCR$\gamma\delta$ mediates recognition of dipalmitoyl PC coated to CD1c, a series of mAb blocking experiments was conducted using mAbs against TCR$\alpha\beta$ and TCR$\gamma\delta$ to test their ability to block proliferation of D5/5.10 T cells. Only the mAb against TCR$\gamma\delta$ blocked proliferation (by 75%), suggesting that CD1c-restricted PC recognition was TCR mediated (Fig. 1*D*). Similar results were obtained for all TCRγd^+ T cell clones illustrated in Table I with a percentage of proliferation inhibition ranging from 68 to 72%.

Of the four DN TCR$\gamma\delta^+$ T cell clones (Table I), three were reactive with both autologous APCs and C1R transfectants (D3/7.8, D9/4.14, and D10/9.3) and recognized synthetic, as well as natural PLs extracted from brain, liver, and chicken egg. According to the results obtained, saturated PC (16:0/16:0) and unsaturated PG (16:0/18:2) seems to be the lipid Ags to which these γδ T cell clones were reactive. In contrast, another DN TCR$\gamma\delta^+$ T cell clone (D5/9.7) reacted with unsaturated PE (18:2/18:2) and was CD1c restricted. Such unsaturated PL belongs to several natural sources, like exogenous soy or pollen extracts, where both saturated and unsaturated species are present in different proportions. Moreover, saturated 16:0/16:0 PC and 16:0/18:2 PG are known constituents of several mammalian tissues, and thus may represent autologous Ags expressed preferentially at the surface of $CD1a^+$ (clone D10/9.3), $CD1c^+$ (clone D9/4.14), or $CD1d^+$ (clone D3/7.8) APCs. As experiments conducted seem to indicate, in TCR$\gamma\delta^+$ clones that were reactive with autologous APCs, adding particular PL compounds to diverse $CD1^+$ APC is capable of stimulating proliferation and cytokine secretion (mainly IFN-γ) of responding TCR$\gamma\delta^+$ T cells.

The CD1d-restricted $CD4^+V\delta1^+$ D3/1.8 T cell clone reacted principally with unsaturated synthetic 18:2/18:2 PE, but showed also proliferative responses to natural pollen or soy PE extracts, with an in vitro secretion of IL-4 and IFN-γ. In this case, the exogenous stimulating Ag was the 18:2/18:2 PE, which is well represented in pollen membranes or soybean extracts, as previously reported (16, 17).

Reactivity of duodenal TCR$\alpha\beta^+$ T cell clones

Less than 4% of the 78 TCR$\alpha\beta^+$ T cell clones showed CD1-restricted reactivity toward PLs (Table I and data not shown). One of these (clone D5/9.5) displayed the $CD8^+$ phenotype and was specific for 16:0/18:2 PE, CD1a-restricted and secreted large

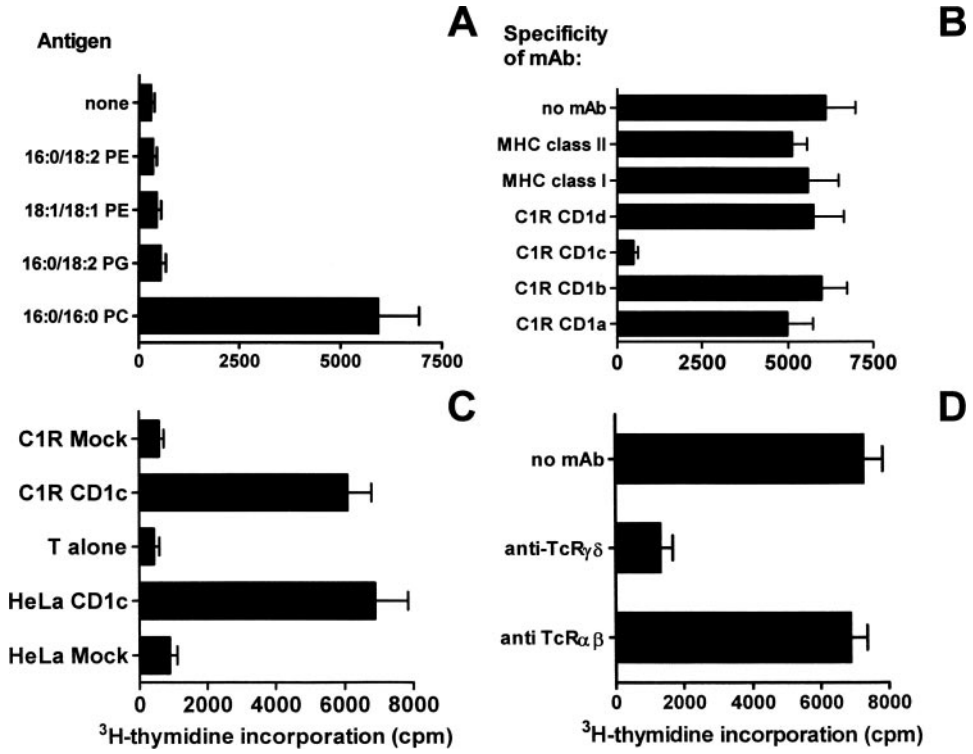

FIGURE 1. Ag specificity of TCR$\gamma\delta^+$ T cell clones. *A*, Recognition of PL Ags. *B* and *C*, CD1 restriction of PL Ag recognition. *D*, Involvement of $\gamma\delta$ TCR in lipid Ag recognition. The experiment shown is representative of similar experiments performed on all TCR$\gamma\delta^+$ T cell clones obtained that showed CD1-restricted PL recognition. Duodenal $\gamma\delta$ T cell clones were obtained as mentioned in *Materials and Methods*. Irradiated (5000 rad) CD1-transfected C1R and mitomycin-C-treated HeLa cell lines were preloaded with lipid Ags and adopted in all experiments as APCs (5×10^4/well). Proliferation of each T cell clone added to the culture (5×10^4/well) was recorded after 5 days of culture by adding 1 μCi of [^{3}H]thymidine. All experiments were performed in triplicate. Synthetic 16:0/16:0 PC was the main PL compound recognized by $\gamma\delta$ T cell clones in a CD1c-restricted manner (*A* and *B*). The specificity of such recognition was confirmed by adding blocking mAbs to CD1 epitopes (*B*) and by using C1R or HeLa transfectants expressing CD1c as APCs (*C*). Lipid Ag recognition involved the $\gamma\delta$ TCR as demonstrated by inhibition of response by preincubation with a mAb specific for TCR$\gamma\delta$ (*D*).

amounts of IL-4 after in vitro stimulation with the specific PL. Twelve other $\alpha\beta^+$ T cell clones, two of which were CD4$^-$ CD8$^-$Vα24$^+$, which is consistent with the phenotype of CD1d-restricted NKT cells with invariant TCRα chains (iNKT cells) (16), reacted principally with C1R transfectants in the absence of exogenously added PLs. Addition of synthetic PLs did not increase

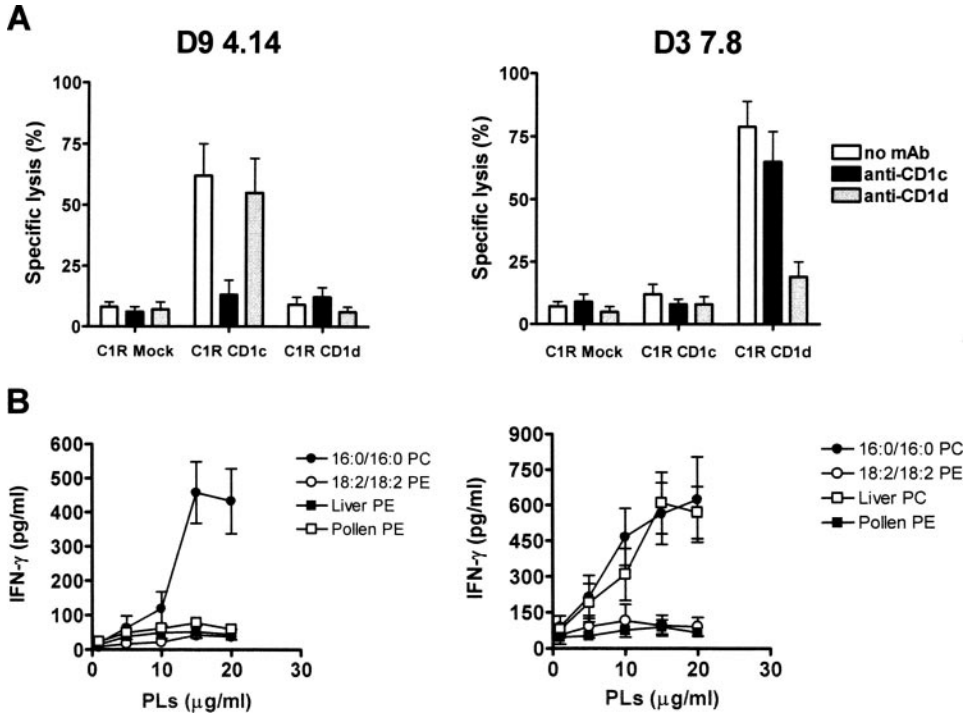

FIGURE 2. Th1 functional activity of duodenal TCR$\gamma\delta^+$ T cell clones. *A*, Cytolytic activity of $\gamma\delta$ T cell clones against CD1c$^+$ and CD1d$^+$ targets. *B*, In vitro production of IFN-γ after PL Ag stimulation. *A*, C1R transfectants were ^{51}Cr labeled and tested (E:T ratio of 30:1 is depicted) for cytolytic assays with D9/4.14 and D3/7.8 T cell clones. The specificity of cytolytic activity in tested clones was confirmed by blocking specific CD1 receptors with mAbs. *B*, The same clones were incubated with C1R transfectants (C1R CD1c for D9/4.14 (*left*) or C1R CD1d for D3/7.8 (*right*)) preloaded with synthetic or natural PLs to assess their ability to secrete Th1 or Th2 cytokines after 24 and 48 h of culture. Whereas IL-4 was undetectable after culture times (data not shown), there was a considerable IFN-γ production by tested clones after stimulation with both synthetic and natural PLs.

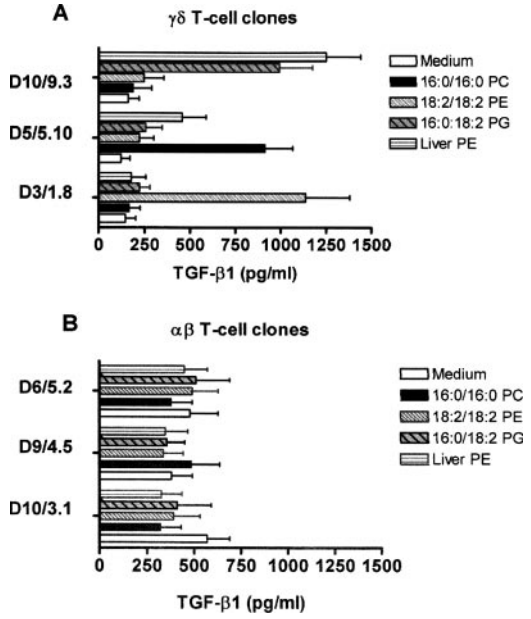

FIGURE 3. Regulatory function of duodenal T cell clones. Three different TCR$\gamma\delta^+$ CD1-restricted and PL-specific clones (*A*: clones D10/9.3, D5/5.1, and D3/1.8) and three TCR$\alpha\beta^+$ CD1-restricted but not PL-specific clones (*B*: CD4$^+$ D10/3.1, Vα24$^+$ D9/4.5, and D6/5.1) were assayed for in vitro production of TGF-β1 and IL-10. All clones were incubated with the relevant CD1 transfectants to which they were reactive (as shown in Table I) plus synthetic and natural PLs for 48 h. Note that all TCR$\gamma\delta^+$ T cell clones showed an Ag-specific variation in TGF-β1 production when compared with unstimulated cultures, whereas TCR$\alpha\beta^+$ T cell clones produced identical levels of the cytokine whether or not the exogenous PLs were added. No specific variations in IL-10 production were recorded for either group of T cell clones (data not shown).

proliferation by either line (data not shown). The remaining 67 TCR$\alpha\beta^+$ T cell clones did not display any reactivity toward PLs and/or CD1$^+$ APCs (data not shown).

Th1-like functional activity of TCR$\gamma\delta^+$ T cell clones

Because it is known that many TCR$\gamma\delta^+$T cell lines are capable of cell-mediated cytolysis (18), DN D3/7.8 and D9/4.14 T cell clones were examined for their ability to lyse transfected C1R lymphoblastoid cell lines C1R CD1d, C1R CD1c, or C1R mock. These T cells failed to lyse C1R mock targets, but efficiently lysed C1R CD1d and CD1c targets (Fig. 2). In each case, lysis was specifically blocked by specific anti-CD1 mAbs. Thus, TCR$\gamma\delta^+$ clones D3/7.8 and D9/4.14 were also CD1d and CD1c reactive, as was also revealed in the proliferation and cytolytic assays with CD1 transfectants cell lines. At the same time, such clones showed in vitro IFN-γ production after stimulation with exogenous synthetic 16:0/16:0 PC. Remarkably, the DN D3/7.8 clone was also responsive to liver PC, with dose-dependent IFN-γ production, thus confirming their prevalent Th1-like function.

Regulatory function among duodenal PL-reactive T cell clones

Because most of IEL are now believed to act as regulatory TCR$\alpha\beta^+$ or TCR$\gamma\delta^+$ T cells at the mucosal surfaces (19), we measured the amounts of TGF-β1 or IL-10 secreted by some clones in response to CD1 transfectants loaded or not with synthetic and natural lipids. We could observe a strong TGF-β1 secretion in culture supernatants of selected TCR$\gamma\delta^+$ T cell clones exposed to synthetic 16:0/16:0 PC, 18:2/18:2 PE, and 16:0/18:2 PG (Fig. 3*A*). No significant variations were recorded for IL-10 secretion in the same experimental conditions (mean, 3.5 ± 1.2 pg/ml in supernatants from unstimulated cultures vs 5.4 ± 3.8 pg/ml in PL-stimulated cultures; p = NS). In contrast, when autoreactive D10/3.1 (CD4$^+$TCR$\alpha\beta^+$), D9/4.5 (CD4$^-$CD8$^-$Vα24$^+$), or D6/5.2 (CD4$^-$CD8$^-$Vα24$^+$) clones were exposed to the same synthetic or natural PLs (Fig. 3*B*), we did not observe significant differences in cytokine secretion patterns between stimulated and unstimulated samples. It is worth noting, however, that such clones showed a basal secretion of TGF-β1 in the absence of Ag stimulation, along with production of IL-10 in some cases (clone D6/5.2, 370 pg/ml; clone D10/3.1, 159 pg/ml). These TCR$\alpha\beta^+$ T cell clones, which proliferated and secreted TGF-β1 in response to both CD1 transfectants and/or cognate interaction with the CD1-lipid complex, could also produce discrete quantities of IFN-γ in vitro. However, we were unable to identify their fine antigenic specificity and/or lipid cellular targets.

Discussion

In the present study, we have described and functionally characterized T cell clones derived from duodenal mucosa of normal donors that recognize CD1 molecules and secrete regulatory cytokines, such as TGF-β1. Among them we were able, for the first time, to demonstrate the existence of a significant fraction of CD1-reactive TCR$\gamma\delta^+$ T cells that depend for their functional activation on the presence of PL Ags, as well as other TCR$\alpha\beta^+$ T cells that recognize CD1 molecules in a manner apparently not dependent on exogenously added lipids. These phenotypically and functionally distinct subsets may represent not only directly reactive T cells that are a part of the innate immune system, but also "regulatory" TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ T lymphocytes devoted to the control of host autoreactivity.

The majority of IEL accumulating in the duodenal mucosa of normal subjects are represented by TCR$\alpha\beta^+$ T cells, with a few TCR$\gamma\delta^+$ lymphocytes present in the lamina propria (20). Most of these TCR$\alpha\beta^+$ T cells are known to express the CD161 NK cell marker and display IFN-γ and TNF-α secretion after in vitro stimulation with ionomycin. Our data substantially confirm these observations, since the majority of our clones were of TCR$\alpha\beta^+$, displayed a prompt IFN-γ secretion capacity, and responded to CD1 transfectants in the absence of foreign lipids.

However, we have observed for the first time that in some cases PHA-derived CD4$^+$TCR$\alpha\beta^+$ and CD8$^+$TCR$\alpha\beta^+$ T cell clones can also respond vigorously to exogenous synthetic and/or natural PLs with detectable IL-4 (e.g., in the case of the CD8$^+$ D5/9.5 T cell clone) and TGF-β1 production. Numerically, such response is limited to a very small fraction of clones among TCR$\alpha\beta^+$ IELs, but this can represent a controlled adaptive response to lipid Ags derived from commensal bacterial flora of the gut and/or lipid components of ingested food, and thus may constitute a specialized T cell subset devoted to the control of host mucosal reactivity (19). Most of the CD1-restricted duodenal IEL T cell clones isolated in the current study were distinct from the well-described CD1d-restricted NKT cell population (iNKT cells) that responds to α-galactosylceramides and has been extensively analyzed in mice and humans. Thus, the majority of our duodenal IEL clones did not express the Vα24$^+$ invariant TCRα chain which is characteristic of iNKT cells, and many were restricted by CD1a or CD1c rather than CD1d.

In the small intestine of humans, mice, chicken, and cattle, TCR$\gamma\delta^+$ T cells comprise a discrete fraction of intestinal intraepithelial lymphocytes. It is now generally accepted that mucosal TCR$\gamma\delta^+$ T cells, owing their limited TCR diversity, contribute to

the fast-acting local innate response, implying that large numbers of TCR$\gamma\delta^+$ T cells share the capacity to respond to the same set of Ags, rather than showing the fine Ag specificity that is characteristic of TCR$\alpha\beta^+$ T cells. However, to date, no antigenic specificities have been demonstrated for gut-associated TCR$\gamma\delta^+$ T cells.

It has been hypothesized that, by responding to self-Ags expressed by infected or transformed epithelial cells, TCR$\gamma\delta^+$ IELs might provide a first line of defense against infectious pathogens attacking the surface of the body. More recently, it was noted that some V$\delta1^+$TCR$\gamma\delta^+$ T cells may be specific for CD1c molecules expressed by APCs, providing the immune system with the capacity to respond rapidly to nonpolymorphic molecules in the absence of foreign Ags, that may activate or eliminate APCs (21). These cells may recognize an autologous self-lipid presented by CD1c, such glycosylphosphatidylinositols that are reported to bind murine CD1 molecules (22).

The recognition of CD1c and CD1d by human TCR$\gamma\delta^+$ T cells may be an important form of Ag recognition by these T cells. CD1 recognition in the absence of foreign Ag could be compared with the alloreactive and autoreactive recognition of MHC class I and II proteins by TCR$\alpha\beta^+$ T cells that are easily detected in MLR and autologous MLR (23). Thus, autoreactivity to restricting elements may be a common theme between TCRs that recognize foreign Ags in the context of MHC and CD1.

CD1 molecules can be directly induced on monocytes as they differentiate into dendritic cells in response to GM-CSF or indirectly in response to agents such as bacteria or inflammatory products that induce the secretion of GM-CSF. Such de novo expression of CD1 can be seen on dendritic cells in granulomas induced by *Mycobacterium leprae* infection in leprosy (24). This new expression of CD1 or stress-induced endogenous lipid Ags presented by CD1 could activate V$\delta1^+$ T cells that are specific for CD1 isoforms. The evidence that each CD1 isoform thus far studied activates some T cells in the absence of added foreign lipids is compelling (25–27), as well as the fact that such self-reactivity is associated with effector functions critical to rapid antimicrobial immune response. The CD1-restricted diverse TCR population probably comprises both conventional naive T cells capable of adaptive responses to foreign lipids and memory/effector T cells capable of both self-reactivity and foreign lipid reactivity, as recently demonstrated in normal human peripheral blood-derived T cell clones (28). Similar conclusions can be argued also from the present study, in which we demonstrate that exogenous PLs can activate CD1-restricted normal duodenal $\gamma\delta$ T cells. Moreover, we also noted that different CD1 molecules can present the same lipids to different $\gamma\delta$ T cell clones, as in the case of CD4$^-$CD8$^-$V$\delta1^+$ D3/7.8 (CD1d) and CD4$^+$V$\delta1^+$ D5/5.10 (CD1c); although from an evolutionary point of view, CD1d and CD1c are quite divergent. This suggests that the presentation of an overlapping set of ligands may be a common rule for CD1-expressing APCs (29). A rationale for maintaining different CD1 molecules with similar lipid-binding specificities would be the pattern of tissue distribution or perhaps the sampling of lipids from different endosomal compartments, rather than the ability to present different lipids (30).

The functional relevance of "regulatory activity" by CD1-restricted $\gamma\delta$ T cells could be proposed not only because of their cytokine production after priming with stimulating lipids, but also on the basis of their ability to lyse cellular targets expressing surface CD1c and CD1d isoforms. Danger signals could up-regulate these molecules at the surface of microbe-infected cells, stressed mucosal epithelial cells (31), transformed tumor cells, or APCs loaded with autologous PLs, underscoring the role of CD1-restricted $\gamma\delta$ T cells in immunosurveillance.

In conclusion, we have provided evidence that a substantial percentage of TCR$\gamma\delta^+$ and, to a lesser extent, of TCR$\alpha\beta^+$ IELs from normal human duodenal mucosa are able to recognize exogenous PLs in a CD1-restricted fashion and can therefore specifically proliferate and secrete regulatory cytokines, such as TGF-β1. This adaptive response raises the intriguing question of whether an inherited and/or acquired imbalance of such a mechanism (e.g., the recently suggested link between western lifestyle, high dietary fat intake, and allergy) (32) could lead to a shift toward polarized Th1 or Th2 effector cells (33), favoring the emergence of inflammatory or allergic intestinal diseases.

Disclosures

The authors have no financial conflict of interest.

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