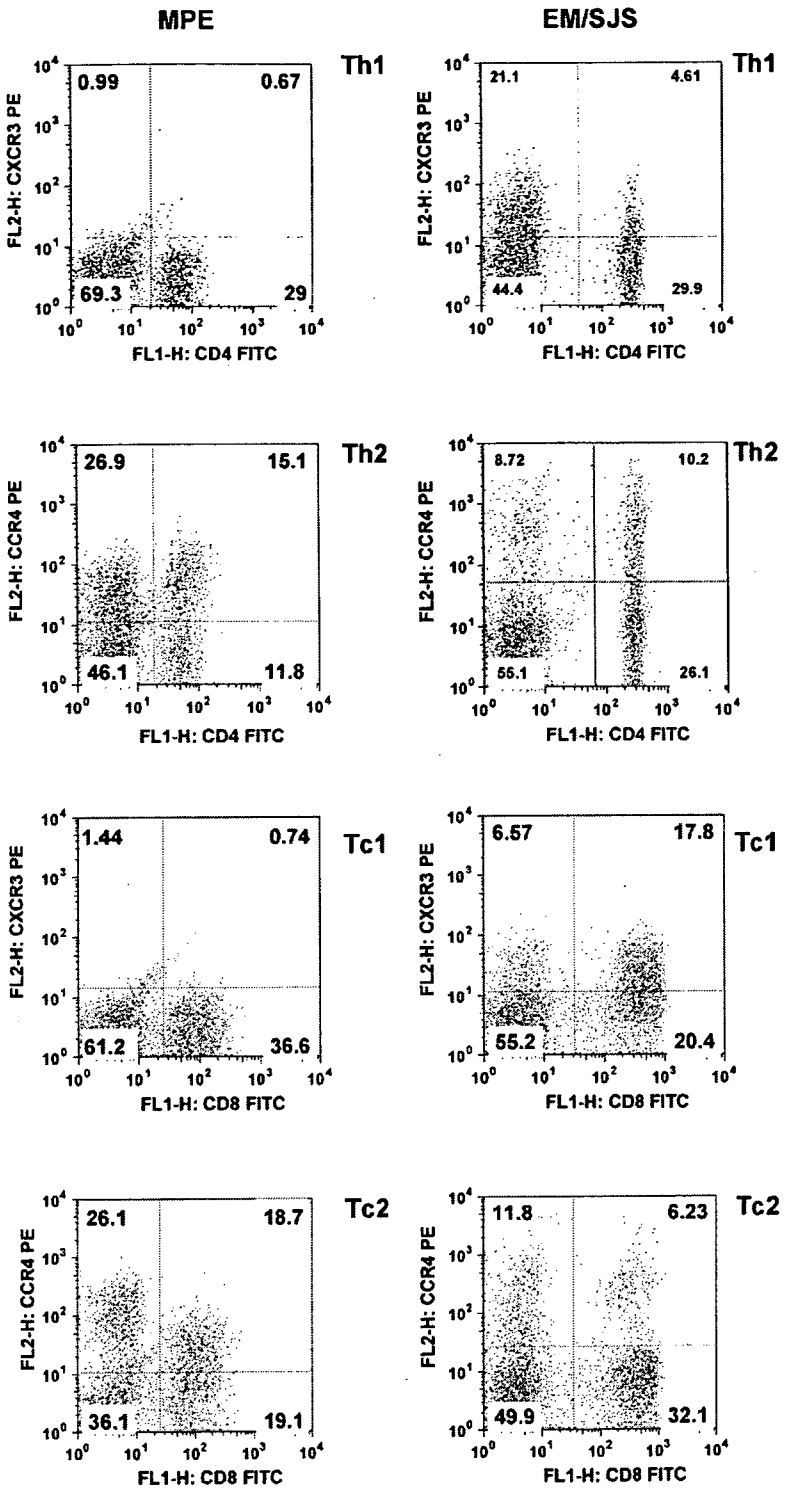

Fig. 2 Flow cytometric analysis of Th1, Th2, Tc1 and Tc2 cells in representative cases of MPE, EM/SJS and viral eruption. The indicated numbers represent the percentage of cells.

"recovery" represents the resolving time point, i.e. 4–6 weeks after the recurrence.

From the onset to the recurrence, $CD8^+$ cells increased and $CD4^+$ cells decreased in all patients (Fig. 4a). The reduction in HLA-DR-positive, activated $CD4^+$ T cells was particularly dramatic (Fig. 4b). While neither $CD4^+$ nor $CD8^+$ cells expressed CD69 (Fig. 4c), $CD4^+$ but not $CD8^+$ cells increasingly expressed CD25 (Fig. 4d). Along with the elevation of $CD8^+$ cells (Fig. 4a), Th1 and Tc1 cells were increased,

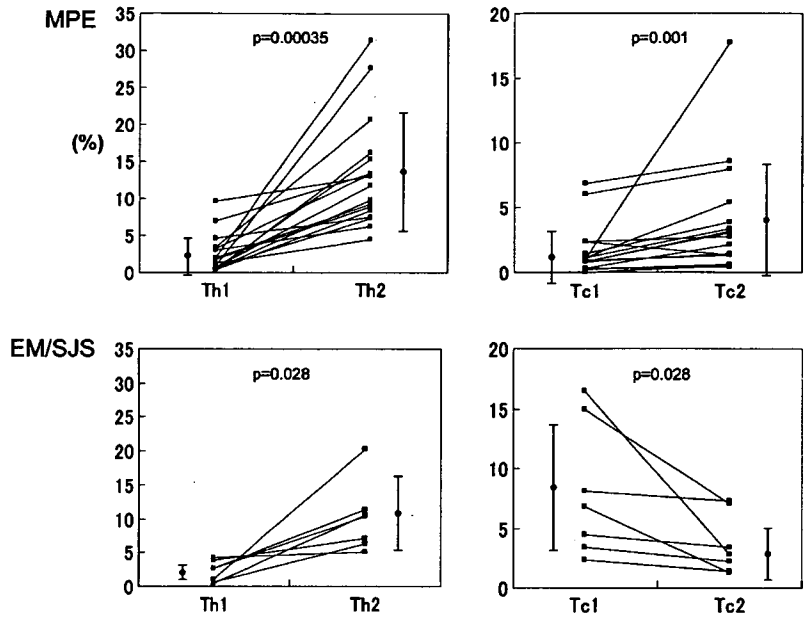

Fig. 3 Percentages of Th1, Th2, Tc1 and Tc2 cells in each patient with MPE or EM/SJS. Vertical bars represent the mean ± S.D.

while Th2 and Tc2 cells were decreased or unchanged (Fig. 4e and f). Therefore, DIHS was characterized by the activation of Th1/Tc1 cells, which were increased at the recurrence stage and prolonged thereafter.

4. Discussion

The present study provides several important findings on the numerical changes in peripheral T cell populations upon occurrence of drug eruptions. It should be careful that the increased T cell populations observed here were not necessarily T cells reactive with a culprit drug. Rather, they might include T cells expanding as a result of the cytokine burst induced by genuine drug-reactive T cells. Furthermore, if some virus is reactivated, resultant stimulation of Tc1/Th1 cells might influence the results. Although the obtained data reflect all of these events, one can evaluate some drug-induced T cell polarization.

There have been controversial findings in the T cell populations responsible for the development of drug eruptions. Previous studies have suggested that the proliferating cells at the acute phase of both MPE and SJS/TEN are Th1 cells by cytokine analysis [8,9]. However, Th2 cells infiltrate in the skin lesions of MPE [17], and β-lactam-specific T cells are Th2 cells [10]. In both MPE and EM/SJS, our study demonstrated that Th2 cells were the propagating population compared to Th1 cells. This is consistent with the infiltration of eosinophils in some of the cases of both types. In contrast, Tc1 and Tc2 cells differentially predominated in EM/SJS and MPE, respectively. The role of Tc2 cells in the pathogenesis of MPE remains speculative, and there have been no report on the presence of Tc2 cells that are involved in the pathogenesis of drug eruptions. It is possible that they propagated in response to a culprit drug, or alternatively, Tc2 cells might merely proliferate as a result of the Th2 cytokine-dominant environment.

The Tc1 dominancy observed in EM/SJS is not a surprising finding, because Tc1 cells play a pivotal role in EM/SJS by attacking epidermal cells [18]. Furthermore, HLA-DR$^+$CD8$^+$ activated T cells were increased in EM/SJS, providing another supportive evidence for Tc1 stimulation in this type of drug eruption. In accordance with our finding, it was reported that a predominant CD8$^+$ T cell activation leads to more severe (bullous) skin symptoms or liver involvement, while predominant activation of CD4$^+$ cells elicits mainly maculopapular reactions [19]. Nevertheless, it is not easy to explain the cooperation of Tc1 and Th2 cells, because of the unmatched cytokine profile. In accordance of this finding, drug-reactive T cell clones or lines generated from SJS/TEN patients were mostly Th2 cells [20]. In our study, 2 (cases 18 and 22) of 7 patients with EM/SJS histologically exhibited both satellite necrosis of epidermal cells and marked infiltration of eosinophils. Such remarkable eosinophilic infiltration has been reported in some patients with EM/SJS [21]. These reported findings, together with the present observation, suggest that EM/SJS involves both the cytotoxic event to the epidermis by Tc1 cells and the allergic reactions to drugs by Th2 cells.

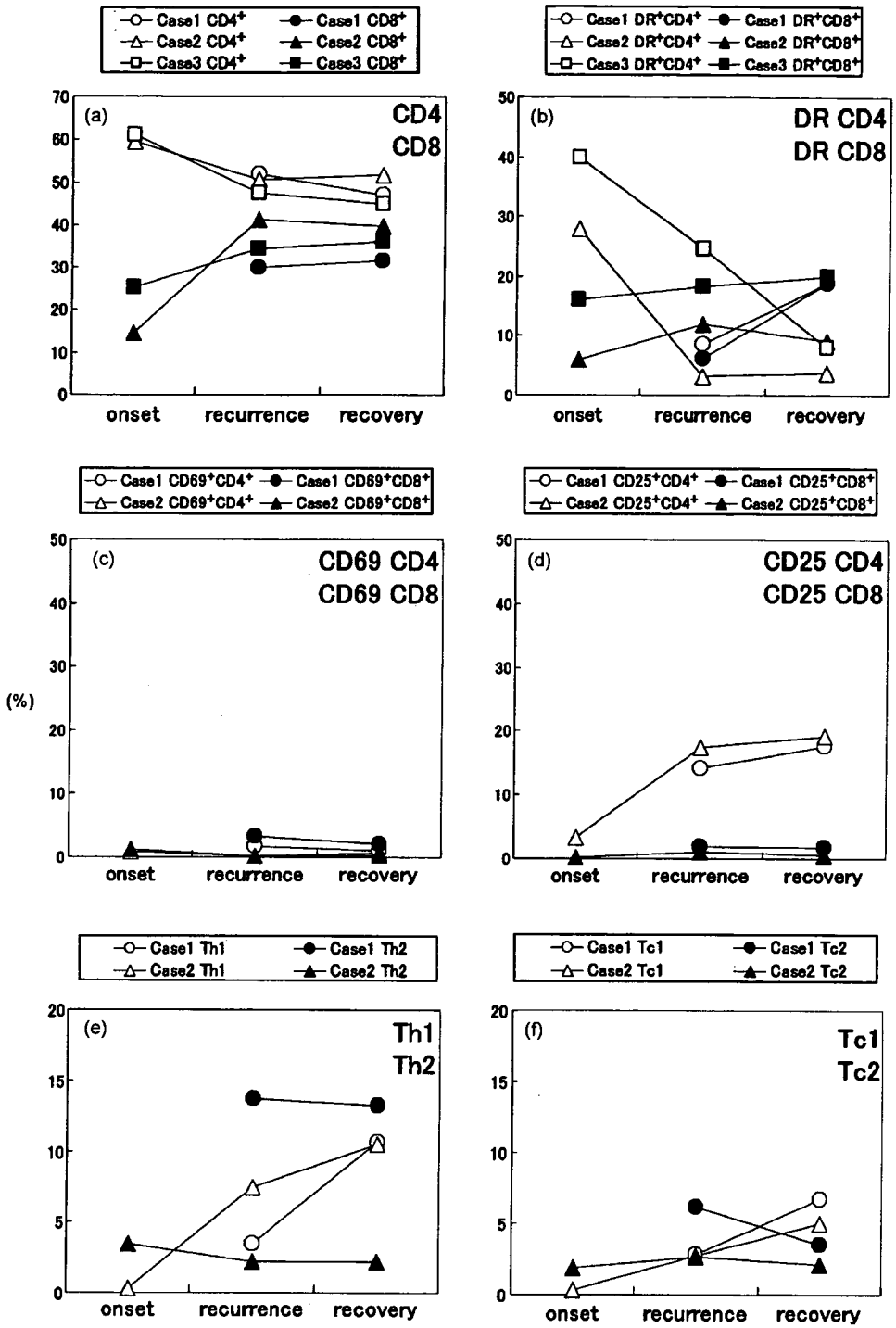

Fig. 4 Changes in the percentages of T cell subsets in patients with DIHS. In cases 25–27, the initial blood examination (onset) was performed when they were not treated, and the second blood samples (recurrence) were taken during treatment with prednisolone, 10–20 mg daily. At the third time point (recovery), the patients did not have oral prednisolone.

In drug eruption to phenobarbital, both $CD4^+$ and $CD8^+$ T cells possessing T cell receptor (TCR) Vβ5.3 are stimulated by this drug [20,22], suggesting the lack of requirement of MHC molecules for presentation or supporting the pharmacological interaction concept between drugs and TCR [23]. This may provide evidence for the dual activation of Th2 and Tc1 cells in EM/SJS.

In DIHS/DRESS, each T cell population was differentially activated, depending on its clinical stage.

From the onset to the second development of the eruption and/or liver dysfunction, the total percentage of $CD8^+$ cells was increased, whereas that of $CD4^+$ cells was decreased. During this clinical course, Tc1 cells were increased as well as Th1 cells, while Th2 and Tc2 cells were decreased or unchanged. $CD4^+$ and $CD8^+$ T cells might respond to a causative drug and virus-infected cells, respectively. Given that $CD4^+$ T cells reactive with a culprit drug are Th2 cells, the Th2 dominant cytokine environment might allow HHV6 or other viruses to be reactivated as a result of suppression of cytotoxic immunity against virus-infected cells. Subsequently, the virus reactivation might induce the activation of Tc1 cells and the production of Th1 cytokines to eliminate virus-infected cells.

As for the early activation markers, CD69 was not expressed in any of the types. This is in accordance with the previous observation that viral eruptions but not drug eruptions exhibit CD69 expression [9]. In all three types of drug eruptions, considerable numbers of $CD25^+CD4^+$ T cells appeared in the blood. It is an important issue whether these cells are activated T cells or Treg. In our study, CTLA-4 was not expressed in any of 3 cases examined. Further studies are necessary for the characterization of $CD25^+CD4^+$ T cells.

Flow cytometric analysis of peripheral blood lymphocytes is one of the diagnostic tools for drug hypersensitivity. Infectious exanthema is a potential differential diagnosis for drug eruptions, especially MPE. Since the viral and bacterial eruptions stimulate Th1/Tc1 or Th0 cells, as has been reported [9] and observed by us in measles and varicella (data not shown), the Th2/Tc2 increasing moiety of MPE is strikingly different. Likewise, the early eruptions of EM/SJS and DIHS can be distinguished from infectious eruptions by the Th2 dominant activation of T cells. The analysis of peripheral T-cell populations provides important information on the differential diagnosis of drug eruptions from infectious exanthema.

Acknowledgements

This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports and Technology in Japan (no. 18591267).

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Int. J. Cancer: **121**, 2585–2590 (2007)

FAST TRACK

Augmented expression of programmed death-1 in both neoplastic and non-neoplastic CD4$^+$ T-cells in adult T-cell leukemia/lymphoma

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Adult T-cell leukemia/lymphoma (ATL) is a CD4$^+$CD25$^+$ T-cell malignancy infected with human T-cell leukemia virus type-I (HTLV-I). HTLV-I infection causes the T-cell dysfunction, which contributes to the immunodeficient state of the patients. Programmed death-1 (PD-1) can negatively regulate T-cell response, when its ligand, PD-L1 or PD-L2 mainly expressed on antigen presenting cells, binds to this B7 family receptor. We investigated whether PD-1 is expressed on CD4$^+$ neoplastic (and/or non-neoplastic) cells or CD8$^+$ cytotoxic cells in peripheral blood mononuclear cells from 11 patients with ATL. By flow cytometry, we found that the levels of PD-1 expression on both CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T-cell populations were increased in ATL patients compared to normal healthy volunteers, while PD-1 levels on CD8$^+$ T-cells were comparable between the patients and normal subjects. In stimulation with anti-CD3 antibody, the proliferation of PD-1-expressing T-cells from ATL patients was weak when compared to that of PD-1-nonexpressing normal T-cells. In addition to PD-1, PD-L1 was coexpressed on ATL cells in some patients, and PD-L1 expression was enhanced by stimulation with anti-CD3 antibody. Finally, the production of cytokines such as TNF-α by ATL cells was restored by blockade of PD-1/PD-L1 interaction. These findings suggest that CD4$^+$ T-cells are the main PD-1-expressing cells rather than CD8$^+$ T-cells in ATL patients, and both neoplastic and normal CD4$^+$ cells are exhausted as a result of PD-1 expression, and additionally PD-L1 expression on the neoplastic cell.

Key words: HTLV-I; ATL; PD-1; PD-ligand

Adult T-cell leukemia/lymphoma (ATL) is a malignancy of mature CD4$^+$ T-cells caused by the human T-cell leukemia virus type-I (HTLV-I).[1,2] The neoplastic T-cell is usually identified with the phenotype of CD4$^+$CD25$^+$CCR4$^+$ cells.[3–5] This endemic hematologic neoplasm develops in 1–5% of individuals infected with HTLV-I after more than 2 decades of viral persistence.[6,7] Based on the organ involvement and severity, ATL is divided into 4 clinical categories: acute, chronic, lymphoma and smoldering types.[8] In any of these clinical subtypes, ATL patients frequently lapse into a severe immunocompromised state, which easily leads to opportunistic infection.[9] In fact, peripheral blood mononuclear cells (PBMC) obtained from ATL patients are unresponsive *in vitro* to T-cell receptor (TCR) stimulation compared to normal healthy donors.[10]

T-cell activation requires the coordinated engagement of multiple receptors expressed on both T-cells and antigen presenting cells (APC). Binding of the TCR to specific peptide-MHC complexes and costimulation of the T-cell through the CD28 receptor are important for the activation of T-cells. CD28 is stimulated by 2 ligands on the APC, CD80 and CD86, which provide signals that enhance T-cell proliferation, cytokine production and survival.[11] On the other hand, cytotoxic T lymphocyte associated antigen-4 (CTLA-4) also interacts with CD80 and CD86 but has inhibitory effects on T-cell function and cell cycle progression.[12,13]

Programmed death-1 (PD-1), a CD28 homologue, contains 2 immunoreceptor tyrosine-based motifs that are phosphorylated upon receptor engagement and recruit Src homology 2-domain-containing tyrosine phosphatase 2.[14–16] In addition, 2 new members of the B7 family, B7-H1 (PD-L1) and B7-DC (PD-L2), have been identified as ligands for PD-1. The binding of PD-L1 or PD-L2 to PD-1 inhibits TCR-mediated T-cell proliferation and cytokine production, leading to the downregulation of T-cell responses.[17–19] The PD-1/PD-L pathway contributes directly to T-cell dysfunction, and this new concept has recently been applied to the mechanism underlying chronic viral infection. For example, PD-1 expression is elevated in human immunodeficiency virus-specifc CD8$^+$ T-cells, and blockade of PD-1/PD-L interaction reverses the exhaustion of the CD8$^+$ T-cell and restores cytokine production and proliferation.[20–22]

In our preliminary study, we have examined PD-1 expression on peripheral CD8$^+$ T-cells in patients with ATL to address the mechanism underlying CD4$^+$ neoplastic cell growth. However, we found that PD-1 is expressed at significantly high levels on CD4$^+$ neoplastic and non-neoplastic cells but not CD8$^+$ cells. This urged us to extensively investigate the PD-1/PD-L interaction in ATL cells. Results suggest that PD-1 plays a role for the immunocompromised condition in ATL patients.

Patients, material and methods

Patients and PBMC

Eleven patients with ATL (listed in Table I) and 7 healthy donors as control were enrolled in our study. All patients and normal healthy volunteers were obtained from the Department of Dermatology, University of Occupational and Environmental Health, Kitakyusyu, Japan, and the following study was performed with informed consent. ATL was diagnosed on the basis of clinical features and laboratory findings according to the criteria.[8] All patients had an integration of HTLV-I proviral DNA as assessed by the standard Southern blot analysis[2] in PBMC. Peripheral blood was obtained by vein puncture from the patients and normal subjects with heparin as anticoagulant. PBMC were isolated by the standard Ficoll-Paque (Pharmacia, Uppsala, Sweden) method.

Antibodies and flow cytometric analysis

Allophycocyanin (APC)-labeled monoclonal antibodies (mAb) to CD4 (RPA-T4), fluorescein isothiocyanate (FITC)-labeled mAb to CD25 (2A3) and PD-1 (MIH4), phycoerythrin (PE)-labeled mAb to CD25 (2A3), PD-L1 (MIH1) and PD-L2 (MIH18), and PE Cy7- labeled mAb to CD8 (RPA-T8) were purchased from BD Bioscience (San Diego, CA). FITC or PE-labeled mouse $IgG_{1,\kappa}$ (MOPC-21) was used as isotype-matched control for PD-1 or PD-L1, 2. Crude or cultured PBMC were washed with phosphate-buffered saline (pH 7.4). Hanks' balanced salt solution containing 0.1% NaN_3 and 1% fetal calf serum (FCS) was used as the stain-

Grant sponsor: Ministry of Education, Culture, Sports, Science, and Technology of Japan; Grant numbers: 17790798, 19790809.

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Received 18 May 2007; Accepted after revision 10 July 2007
DOI 10.1002/ijc.23042
Published online 23 August 2007 in Wiley InterScience (www.interscience.wiley.com).

TABLE I - CLINICAL SUMMARY OF THE ENROLLED PATIENTS

Case	Clinical types	Age/sex	No. of WBC (/μl)	No. of circulating lymphocytes (/μl)	No. of atypical cells (/μl)	LDH	sIL-2R	% of CD4 + CD25 + cells	Treatment
1	A	69/M	20,500	11,270	8,405	234	12,493.5	56.6	Radiation
2	A	83/F	17,500	11,375	11,200	329	9,238.1	45.3	None
3	S	72/M	7,000	4,130	1,400	191	1,006.8	59.3	Steroid ointment
4	S	77/M	5,800	1,700	58	220	1,116.2	10.9	VP-16, Radiation
5	S	65/M	7,200	2,010	360	272	628.1	20.2	VP-16, Radiation
6	S	45/F	4,700	1,030	94	368	888.6	27.1	NB-UVB
7	S	71/M	3,200	470	64	219	2,798.5	17.8	Radiation
8	S	88/M	6,600	2,770	1,122	210	578	34.6	Radiation
9	S	81/M	7,800	2,260	312	211	ND	21.0	VP-I6
10	A	55/M	8,000	2,960	2,240	669	11,014.4	59.0	None
11	S	69/F	6,800	2,992	2,108	271	1,571.9	47.5	None

S, smoldering; A, acute; M, male; F, female; LDH, lactate dehydrogenase; sIL-2R, soluble interleukin-2 receptor; ND, not determined; NB-UVB, Narrowband-ultraviolet B; % of CD4′ CD25′ cells in PBMC was determined by flow cytometry.

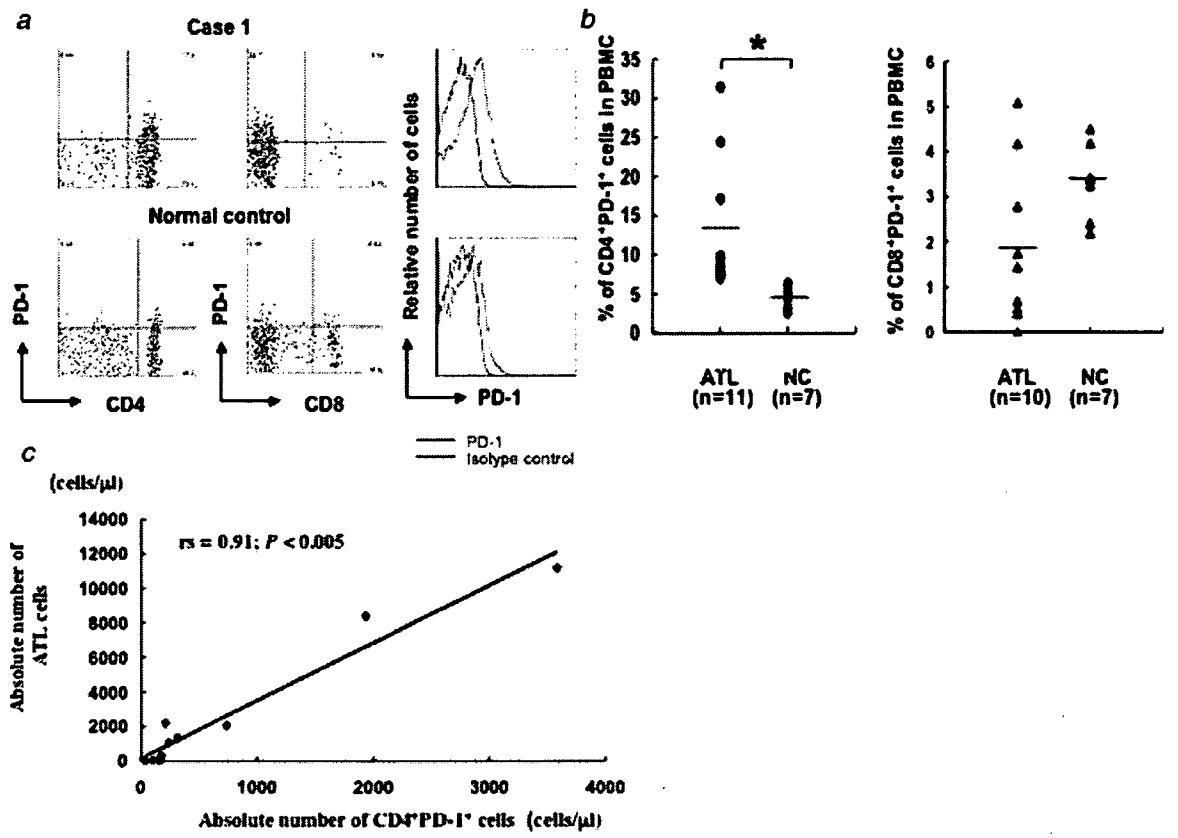

FIGURE 1 – PD-1 expression on $CD4^+$ T-cells of patients with ATL. (*a*) Dot plots showing PD-1 expression on $CD4^+$ T-cells in freshly isolated PBMC of both patient and normal control, and histograms showing the expression of PD-1 in PBMC of the same patient and normal control. The blue line represents the isotype control staining, and the red line represents PD-1 staining. (*b*) Frequency (means ± SE) of $CD4^+PD\text{-}1^+$ or $CD8^+PD\text{-}1^+$ in PBMCs of ATL ($n = 11$) and normal controls ($n = 7$). $^*p < 0.0005$, compared to normal controls. (*c*) Correlation between the absolute numbers of $CD4^+PD\text{-}1^+$ cells and those of circulating ATL tumor cells (rs = 0.91, $^*p < 0.005$).

ing buffer. After incubation on ice for 20 min with mAb or isotype-matched controls, 10,000-labeled cells were analyzed on a FACSCanto (BD Bioscience) in each sample.

Proliferation assay

RPMI-1640 (Gibco BRL Life Technology, Grand Island, NY) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 10^{-5} M sodium pyruvate, 25 mM HEPES, 1% nonessential amino acids and 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Gibco) was used as culture medium. PBMC (1.5×10^5/150 μl of medium per well) were cultured in 96-well U bottom plates (Corning, Corning NY) at 37°C in 5% CO_2 in air in the absence or presence of anti-CD3 mAb (soluble form stimulatory for T-cells; BD PharMingen) at 0.5 μg/ml. After 72 hr culture, their proliferative response was tested by addition of 1 μCi/well of [^{3}H]thymidine (Amersham, Arlington, IL) 16 hr before harvest. The amount of incorporated [^{3}H]thymidine was determined by liquid scintillation spectroscopy. To determine whether the PD-1 expressed on the cells was functional, the

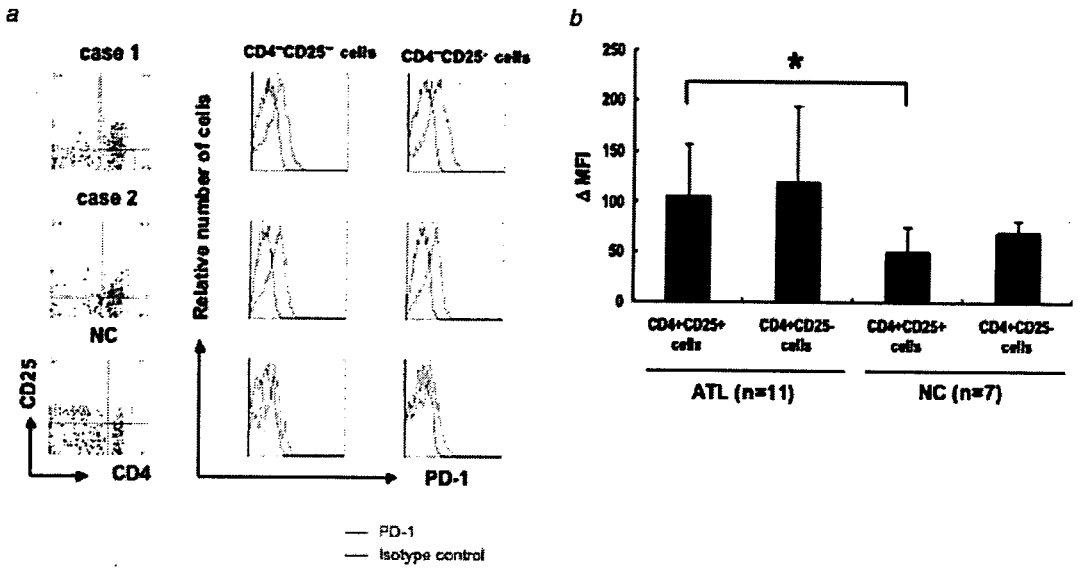

FIGURE 2 – PD-1 expression on $CD4^+CD25^+$ T-cells of patients with ATL. (*a*) Dot plots showing gated $CD4^+CD25^+$ T-cells in freshly isolated PBMCs of both patients and normal control, and histograms showing expression of PD-1 on $CD4^+CD25^+$ T-cells of the same patients and normal control. The blue line represents the isotype control staining, and the red line represents the PD-1 staining. (*b*) Δmean fluorescent intensity (MFI) was calculated as MFI of PD-1-MFI of isotype control. Average of ΔMFI values plus SE for ATL patients ($n = 11$) and healthy controls ($n = 7$) is shown. $*p < 0.05$, compared to normal controls.

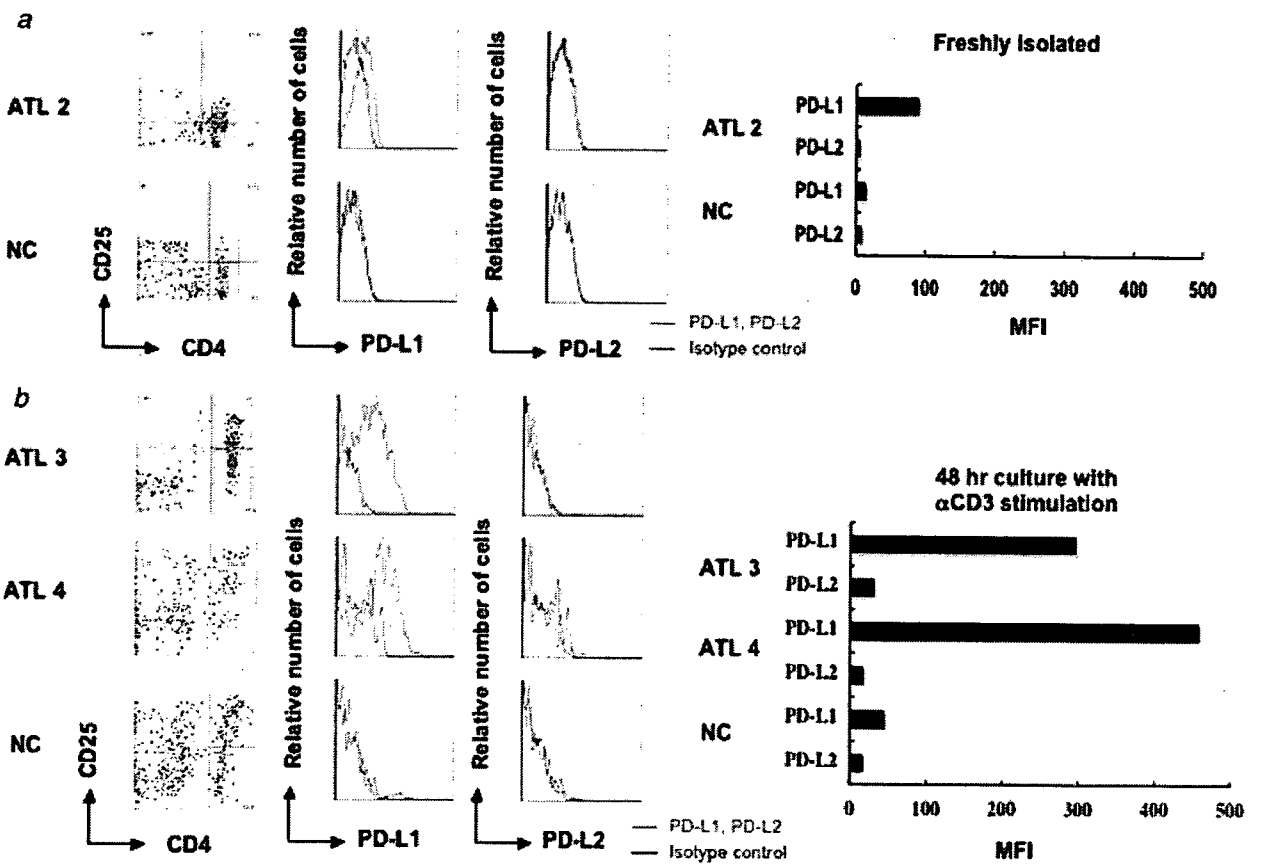

FIGURE 3 – Expression of PD-L1 on neoplastic $CD4^+CD25^+$ T-cells. (*a*) Dot plots showing gated $CD4^+CD25^+$ T-cells in freshly isolated PBMCs of both patients and normal control, and histograms showing expression of PD-L1 or PD-L2 on $CD4^+CD25^+$ T-cells of the same patient and normal control. The blue represents the isotype control staining, and the red line represents the PD-L1, 2 staining. MFI values of PD-L1, 2 for ATL patient and healthy control are shown. (*b*) Dot plots showing gated $CD4^+CD25^+$ T-cells from PBMCs after 48 hr culture with anti-CD3 mAb in both patients (Cases 3 and 4) and normal control, and histograms showing expression of PD-L1 or PD-L2 on $CD4^+CD25^+$ T-cells of the same patients and normal control. The blue represents the isotype control staining, and the red line represents the PD-L1, 2 staining. MFI values of PD-L1, 2 for ATL patient and healthy control are shown.

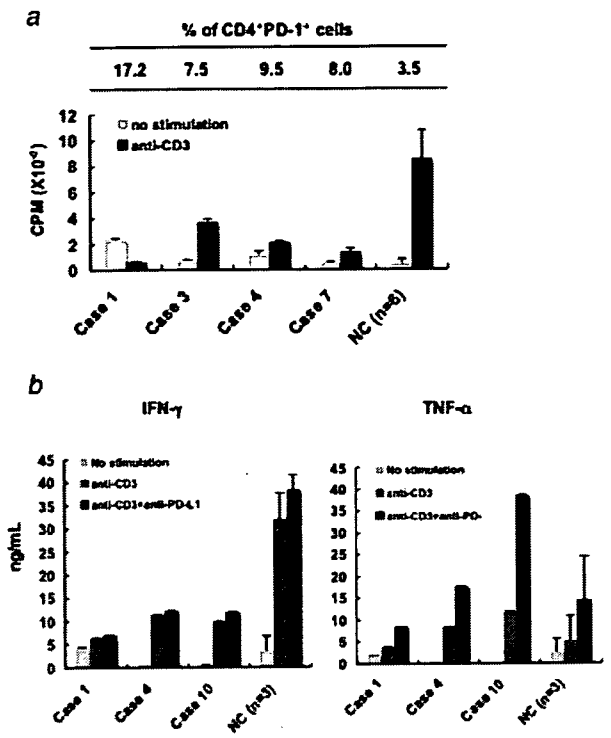

FIGURE 4 – Low response of PBMC from PD-1 highly expressing cases to TCR stimulation. (*a*) Proliferation of PBMC obtained from 4 different ATL patients (Cases 1, 3, 4 and 7) and normal controls (n = 6) after stimulation with anti-CD3 mAb. Bars show mean plus SE from triplicate culture. Percentages of $CD4^+PD\text{-}1^+$ cells in PBMCs of each case and normal controls were indicated upper the column. (*b*) Production of INF-γ and TNF-α in PBMCs obtained from 3 different ATL patients (Cases 1, 4 and 10) and normal controls (n = 3) after stimulation with anti-CD3 mAb, and the effect of anti-PD-L1 mAb.

anti-PD-1 mAb or goat IgG (R&D Systems, Minneapolis, MN), or anti-PD-L1 mAb or mouse IgG_1 (eBioscience, San Diego, CA) was added in the assay at the final concentration of 5 μg/ml.

Cytokine quantification

The concentrations of IFN-γ, TNF-α, IL-4, IL-5 and IL-10 in the supernatants were measured by the Cytometirc Bead Array Kit (BD Bioscience) according to the manufacturer's instructions.

Statistical analysis

Statistical significance was determined by the Mann–Whitney's U test and the correlation between the absolute numbers of $CD4^+PD\text{-}1^+$ and circulating tumor cells was analyzed statistically using Spearman's rank correlaion. p below 0.05 was considered significant.

Results

PD-1 expression on $CD4^+$ T-cells in ATL patients

We first examined the PD-1 expression on $CD4^+$ and $CD8^+$ T-cells obtained from the ATL patients' and normal subjects' PBMC. As represented by Case 1 (Fig. 1*a*), PD-1 expression on $CD4^+$ T-cells was significantly higher in the ATL patient than that in a normal healthy subject. The means ± SE of $PD\text{-}1^+CD4^+$ cell percentage in the patients and normal controls were 12.74 ± 2.47 *vs.* 4.68 ± 0.53 ($p < 0.0005$, Fig. 1*b*). There was a tendency toward lower expression of PD-1 on $CD8^+$ T-cells in most of the patients than that in the controls, but the difference was not statistically significant (Fig. 1*b*). It is notable that PD-1 was highly expressed on $CD4^+$ T-cells in the patients with a highly leukemic state (representative data shown in Fig. 1*a*), and there was a statistically significant correlation (rs = 0.91; $p < 0.005$) between the absolute number of $PD\text{-}1^+CD4^+$ cells and that of circulating atypical cells (Fig. 1*c*).

PD-1 expression on neoplastic $CD4^+CD25^+$ and non-neoplastic $CD4^+CD25^-$ T-cells

ATL tumor cells have a phenotype of $CD4^+CD25^+$ T-cells. We gated on the $CD4^+CD25^+$ or $CD4^+CD25^-$ T-cells and analyzed the mean fluorescent intensity (MFI) of PD-1 and control mouse $IgG_{1,\kappa}$, as represented in Figure 2*a*. ΔMFI was calculated as follows: MFI of PD-1 − MFI of mouse $IgG_{1,\kappa}$. There was no difference in the MFI of PD-1 between $CD4^+CD25^+$ and $CD4^+CD25^-$ T-cells in ATL patients (Fig. 2*b*). PD-1 expression on $CD4^+CD25^+$ T-cells was higher in the patients than the normal controls (means ± SE, 105.8 ± 15.2 *vs.* 50.9 ± 9.05; $p < 0.05$). The similar result was obtained in $CD4^+CD25^-$ T-cells from both groups. These results suggested that not only ATL tumor cells but also non-neoplastic $CD4^+$ T-cells, if infected with HTLV-1, express PD-1.

Expression of PD-L1 (B7-H1) on neoplastic $CD4^+CD25^+$ T-cells

PD-L1 is abundantly expressed in a number of tumor cell lines and various tumor tissues.[23–25] Cancer cells expressing PD-L1 have been shown to promote apoptosis of tumor-attacking T-cells[24] and to inhibit $CD4^+$ and $CD8^+$ T-cell activation.[25] These observations raise a possibility that ATL malignant cells bear PD-L. In a patient with a highly leukemic state (Case 2), $CD4^+CD25^+$ tumor cells expressed a low but discernible level of PD-L1 when freshly isolated PBMC were examined (Fig. 3*a*). Non-neoplastic $CD4^+CD25^-$ cells did not express PD-L1. After incubation with anti-CD3 mAb for 48 hr, other 2 cases (Cases 3 and 4) also expressed high levels of PD-L1 compared to a normal subject (Fig. 3*b*). B7-DC (PD-L2) was not present on $CD4^+CD25^+$ cells in any of the patients or normal healthy controls. Thus, ATL cells express PD-L1 particularly in an activated state.

Low TCR-mediated response in PD-1-highly expressing ATL cases

In PD-1-expressing cells, TCR-mediated T-cell proliferation and cytokine production could be inhibited *via* interaction with PD-L1 or -L2 on APC, leading to the downregulation of T-cell responses.[17–19] We tested PBMC from ATL patients and normal volunteers in their ability to respond to TCR stimulation. As expected, PBMC from some of the patients failed to respond to TCR stimulation compared to normal group (Fig. 4*a*). Notably, the proliferation of PBMC of PD-1-highly expressing case (Case 1) was downregulated by TCR stimulation (Fig. 4*a*). However, this unresponsiveness to TCR stimulation was not restored by the blockade of PD-1 or PD-L1 with a specific mAb (data not shown). We then analyzed the cytokine production by PBMC with or without TCR stimulation. The amount of IFN-γ produced by stimulation was lower in ATL patients than normal subjects, and the blockade with an anti-PD-L1 mAb could not significantly augment IFN-γ production in the patients or controls (Fig. 4*b*). However, the addition of the anti-PD-L1 mAb remarkably increased the production of TNF-α (Fig. 4*b*), suggesting that PD-1/PD-L interaction plays a role in certain outcomes.

Discussion

Our study demonstrated that PD-1 is significantly expressed on $CD4^+$ T-cells but not $CD8^+$ T-cells in patients with ATL. Among $CD4^+$ T-cells, both $CD25^+$ ATL cells and $CD25^-$ non-neoplastic T-cells expressed PD-1. Therefore, $CD4^+$ T-cells rather than $CD8^+$ T-cells are important in the T-cell exhaustion of ATL patients. In lymphoproliferative disorders, there has been only 1

report describing PD-1 expression on tumor cells. In angioimmunoblastic T-cell lymphoma, neoplastic T-cells are immunoreactive for PD-1, but other subtypes of T-cell and B-cell non-Hodgkin lymphoma do not express PD-1.[26] We observed that there was a statistically significant correlation between the absolute number of ATL tumor cells and that of $PD\text{-}1^{+}CD4^{+}$ cells. Thus, PD-1 can be used as a marker for ATL malignant cells as well as CD25 and CCR4.

PD-1 receptor is a member of the B7 family of costimulatory molecules that modulate T-cell antigen-specific receptor signaling and govern T-cell inactivation and survival.[27] Unlike CTLA-4, PD-1 is induced on T-cells, B-cells and monocytes after activation.[28] CTLA-4 and PD-1 are both immunoinhibitory and act on T-cell populations of different avidities. CTLA-4 is most strongly induced and transported to the immunological synapse by a strong TCR signal, and inhibits T-cells that express a TCR with high avidity.[29,30] In contrast, PD-1 is more strongly induced in T-cells that have received a weak TCR signal and most potently inhibits T-cells that have received a weak TCR signal.[18] PD-1 protein can also accumulate within the cytoplasm of natural regulatory T (Treg) cells, becoming upregulated to the cell surface after TCR activation.[31]

Several groups of investigators have postulated the association of ATL tumor cells with Treg cells, because of the $CD4^{+}CD25^{+}CTLA\text{-}4^{+}$ phenotype shared by Treg cells and the marked immunodeficient state of the patients. Most recently, the functional suppressive activity in a cell–cell contact-dependent manner has been demonstrated in both circulating or lymphnode-infiltrating ATL cells and a HTLV-I-infected cell line.[10,32–34] However, it remains controversial whether ATL cells serve as Treg cells, since the cells from only a limited number of patients have been shown to be functional. The present study suggests that $CD4^{+}$ T-cells in ATL patients are in an exhausted state and TCR-mediated responses are downregulated because of the PD-1 expression. The association between the unresponsive state and the immunoregulatory function of the ATL cell is an issue to be further elucidated in future.

In the patient at a highly leukemic stage, ATL tumor cells expressed PD-L1 as well as PD-1, and in the additional 2 cases, PD-L1 was induced by CD3 stimulation. In several malignancies, the PD-1/PD-L pathway plays a pivotal role in tumor evasion from anti-tumor immune responses. In renal cell carcinoma and non-small cell lung carcinoma, PD-L1 expressed on tumor cells may exert anti-tumor activity by inhibiting PD-1-expressing tumor-infiltrating lymphocytes or by inducing their apoptosis.[35,36] In our study, PD-1 was expressed on not only ATL tumor cells but also $CD4^{+}CD25^{-}$ normal T-cells, which are not neoplastic but perhaps infected with HTLV-1. This suggests that PD-L1-bearing ATL tumor cells escape from anti-tumor immunity, where normal $CD4^{+}$ T-cells in collaboration with $CD8^{+}$ cytotoxic T-cells are involved. In addition, our finding may raise a possibility that there exists the combined PD-1 and PD-L1 autocrine loop in ATL cells. Given this possibility, ATL cells might loose the proliferative ability or viability by this loop. In our study, however, PD-L1 was not necessarily produced by PD-1-highly expressing ATL cells, allowing the survival of ATL cells.

In conclusion, we demonstrated the expression of PD-1 on $CD4^{+}$ neoplastic and non-neoplastic T-cells, and that of PD-L1 on the neoplastic cells in ATL patients. It is suggested that the PD-1/PD-L1 pathway may contribute to the marked immunodeficient state of ATL patients.

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Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2006.03258.x

Innate immunity mediated by epidermal keratinocytes promotes acquired immunity involving Langerhans cells and T cells in the skin

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Summary

Skin is an immunological organ consisting of epidermal cells, i.e. keratinocytes and Langerhans cells (LCs, antigen-presenting dendritic cells), and both innate and acquired immune systems operate upon exposure of the skin to various external microbes or their elements. To explore the relationship between innate and acquired immunities in the skin, we investigated whether Toll-like receptor (TLR) ligation of epidermal cells enhances the ability of LCs to present a specific antigen to T cells in mice. LC-containing epidermal cells were incubated with CpG oligonucleotide (TLR9 ligand) modified with trinitrophenyl hapten, and cultured with hapten-primed $CD4^+$ T cells. TLR9 ligand was capable of enhancing the hapten-presenting ability of LCs when LC-enriched epidermal cells, but not purified LCs, were used as the LC source, suggesting that bystander keratinocytes play a role in the enhancement of LC function. Cultivation of freshly isolated epidermal cells with CpG promoted the expression of major histocompatibility complex (MHC) class II and CD86 molecules on LCs. CpG enhanced the production of interleukin (IL)-1α, granulocyte–macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor (TNF)-α by primarily cultured keratinocytes. The addition of a cocktail of neutralizing antibodies against these cytokines abrogated the CpG-promoted, antigen-presenting ability of LC-enriched epidermal cells. Moreover, the addition of culture supernatants from CpG-stimulated keratinocytes restored the ability of purified LCs. Our study demonstrated that although the direct effect of CpG on LCs is minimal, LC function can be up-regulated indirectly by cytokines released by CpG-stimulated keratinocytes. This also implies that innate immunity evoked by TLR ligation of keratinocytes enhances acquired immunity comprising LCs and T cells.

Keywords: antigen presentation, keratinocytes, Langerhans cell, Toll-like receptor

Accepted for publication 16 October 2006
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Introduction

Conserved microbial structures, termed pathogen-associated molecular patterns are recognized by the immune system consisting of TLRs [1,2]. TLRs recognize microorganisms such as cell wall lipid, lipopolysaccharide (LPS), peptidoglycan and CpG oligonucleotide (CpG) [3]. The presence of TLRs on skin resident immune cells may assist in mounting a rapid and efficient host defence against invading pathogens or tissue injury-causing events. TLRs are expressed on epidermal cells (ECs), including keratinocytes [4,5] and Langerhans cells (LCs) [6] and mast cells [7]. It has been shown recently that TLRs expressed on human and murine ECs are functional and play an important part in skin innate immunity [4,6]. Murine LCs have been shown to express mRNA for TLR9 [6], but the expression and function of TLR9 on murine keratinocytes remains controversial.

Although TLRs expressed at sites of host–pathogen interaction probably serve to protect the host from pathogens, unnecessary immune responses to commensal bacteria may harm the host. This concept has been illustrated by several studies of TLR expression in LCs. In comparison with dendritic dells (DCs), LCs respond differently to microbial TLR ligands. For example, LPS is capable of inducing CD83 expression on DCs but not monocyte-derived LC-like cells [8]. Another group of investigators has also demonstrated a

difference in TLR activation between LCs and DCs; stimulation with a ligand for TLR9 matures splenic DCs but not LCs [6]. These findings have suggested that LCs are beneficially low-responsive to skin commensals.

Even if LCs respond poorly to TLR ligands, as assessed by their expression of major histocompatibility complex (MHC) class II and co-stimulatory molecules, this does not exclude the possibility that TLR agonists modify the function of LCs when the skin is exposed to the agonists. Not only LCs but also keratinocytes are involved in the epidermal immunity. The latter cells are a pivotal constituent, participating profoundly in the both innate and acquired immunity [9]. Thus, the possibility arises that the innate immunity evoked via TLRs on keratinocytes enhances the acquired immunity consisting of LCs and antigen-specific T cells. To explore this issue, we investigated whether the ability of epidermal LCs to present a hapten to T cells is altered by CpG oligonucleotide, a TLR9 ligand, in the presence or absence of keratinocytes. Results suggest that the co-existence of LCs and keratinocytes is required for augmentation of LC function upon exposure of ECs to CpG.

Materials and methods

Animals and reagents

Female BALB/c mice, 8 weeks old, obtained from Charles River Japan, Inc., Yokohama, Japan, were maintained in our conventional animal facility. 2,4,6-Trinitrochlorobenzene (TNCB) and 2,4,6-trinitrobenzene sulphonic acid were obtained from Tokyo Kasei, Tokyo, Japan. CpG (ODN 1826) was purchased from Invitrogen (San Diego, CA, USA). As blocking antibodies, purified anti-mouse interleukin (IL)-1α, granulocyte–macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor (TNF)-α monoclonal antibodies (MoAbs) were obtained from BD PharMingen (San Jose, CA, USA).

Culture medium

RPMI-1640 (Sigma-Aldrich, St Louis, MO, USA) was supplemented with 10% heat-inactivated fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 25 mM HEPES, 1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 μg/ml streptomycin.

Flow cytometry

The following MoAbs were employed: phycoerythrin (PE)-labelled anti-mouse I-A^d, fluorescein isothiocyanate (FITC)-labelled anti-mouse CD11c, CD4, CD86 (all from BD PharMingen) and TLR9 (eBiosience, San Diego, CA, USA). All MoAbs were used at 1–5 μg/10^6 cells, and incubation was performed for 30 min at 4°C, followed by two washes in phosphate-buffered saline (PBS, pH 7·4) supplemented with 5% FCS and 0·02% sodium azide. Non-specific stains were performed with the adequate same-class immunoglobulin for specific MoAb. Fluorescent profiles were generated using a FACScan (Becton Dickinson, San Jose, CA, USA). To analyse the expression of MHC class II and CD86 on LCs, ECs were first incubated with anti-mouse Fcγ II/III receptor MoAb for 5 min to prevent non-specific binding of the subsequent reagents to Fc receptors and double-stained with PE-labelled anti-I-A^d and FITC-labelled anti-CD86 MoAbs. Gate was set so that only MHC class II-bearing, size-gated ECs were included in the analysis. 7-Amino-actinomycin D (7-AAD) was added to exclude dead cells.

For intracytoplasmic staining of TLR9, ECs were stained with PE-labelled anti-I-A^d and FITC-labelled anti-TLR9 MoAbs followed by using the Cytofix/Cytoperm plus Fixation/Permeabilization Kit (BD Biosciences, San Diego, CA, USA).

Preparation of LC–ECs and purified LCs

Ears from sacrificed naïve mice were split along the plane of the cartilage, which was then removed together with subcutaneous tissue. The specimens were incubated for 1 h at 37°C in a 0·2% solution of trypsin in PBS. After incubation, the epidermis was separated from the dermis and ECs were dispersed in PBS supplemented with 10% FCS by rubbing the separated epidermal sheets. The cells were filtered and washed twice in PBS.

To examine the effect of TLR9 ligand, EC suspensions were incubated with various concentrations of CpG for 24 h in the culture medium before preparation of LC–ECs. LC–ECs were obtained with Ficoll-Hypaque (specific gravity: 1·083; Sigma-Aldrich, St Louis, MO, USA), as described previously [10]. The percentage of LCs in LC–EC fraction was 15–20%, as assessed by flow cytometric analysis with anti-I-A^d antibody. For further purification of LCs, $CD11c^+$ cells were positively selected from LC–ECs using an anti-CD11c MACS microbead antibody (Miltenyi Biotec Inc., Auburn, CA, USA). The purity of LCs was assessed by flow cytometry after staining with anti-CD11c–FITC and anti-I-A^d–PE MoAbs (BD PharMingen) and their purity was 70–80%.

In vitro proliferation of TNCB-immune T cells to trinitrophenyl (TNP)-modified LC–ECs or purified LCs

Mice were sensitized with TNP hapten by painting 0·05 ml of 5% TNCB in ethanol: acetone (3 : 1) onto the clipped abdomens on day 0. On day 5, lymph node cell (LNC) suspensions were prepared from inguinal and axillary lymph nodes. $CD4^+$ T cells were isolated negatively with a cocktail of conjugated MoAbs (anti-CD8a, CD45R, CD49b, CD11b and Ter-119) (Miltenyi Biotec Inc.), and their purity was > 96%. TNP-modification of LC–ECs or purified LCs was

performed by incubating with trinitrobenzene sulphonic acid as described previously [11]. Immune CD4$^+$ T cells (2×10^5 cells/well) were cultured in triplicate with TNP-modified LC–ECs or purified LCs (5×10^3 cells/well) and various concentrations of CpG in a final volume of 200 µl in 96-well microtitre plates (Corning Glass Works, Corning, NY, USA) for 72 h at 37°C in 5% CO_2. Indomethacin (Sigma-Aldrich, St Louis, MO, USA) was added to the culture at a final concentration of 1 µg/ml. Methyl [^{3}H]-thymidine (Amersham, Arlington, IL, USA) was added (1 µCi/well) 18 h before harvest. The cells were collected on glass fibre filters using a cell harvester (Futaba Medical Inc., Tokyo, Japan) and their radio-uptake was measured in a scintillation counter.

Keratinocyte culture and quantification of cytokines in the supernatants

Freshly isolated BALB/c ECs were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells (7×10^6/well) were then cultured (1·1 ml/well) for 72 h in the presence or absence of CpG in 24-well plates (Corning Glass Works) at 37°C in 5% CO_2. The culture supernatants were collected, stored at −80°C and measured for IL-1α, GM-CSF, and TNF-α by enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analysis

Student's *t*-test was employed to determine statistical differences between means.

Results

TLR9 ligand is unable to stimulate hapten-immune CD4$^+$ T cells in the absence of accessory cells

In advance of investigating the effect of CpG on LCs, we first tested its direct effect on T cells. Crude LNCs or purified CD4$^+$ T cells from TNP-sensitized mice were cultured for 72 h in the presence of various concentrations of CpG. As shown in Fig. 1, LNCs proliferated well in response to CpG, but CD4$^+$ T cells purified from the immune LNCs proliferated only marginally with the same stimulant. Thus, CpG was incapable of stimulating T cells *per se*, but it could activate T cells in the presence of TLR-bearing bystander accessory cells residing in the lymph nodes.

Both keratinocytes and LCs express intracytoplasmic TLR9

It has been reported that LCs express mRNA for TLR9 as assessed by reverse transcription–polymerase chain reaction (RT–PCR) [6] and TLR9 is located in the endoplasmic reticulum in DCs [12]. Although murine keratinocytes were reported not to express TLR9 by RT–PCR [6], we analysed its expression in murine keratinocytes along with LCs by flow cytometry. We could not detect its expression on the surface of keratinocytes or LCs (data not shown), TLR9 was found to be expressed intracytoplasmically in both keratinocytes and LCs (Fig. 2).

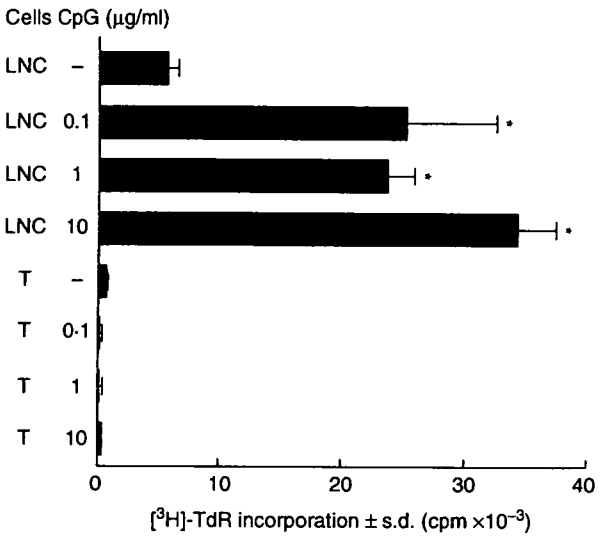

Fig. 1. CpG oligonucleotide (CpG) stimulates crude lymph node cells (LNCs) but not purified CD4$^+$ T cells. 2,4,6-Trinitrochlorobenzene (TNCB)-immune LNCs or T cells were cultured for 72 h with varying concentrations of CpG. $^*P < 0{\cdot}01$, compared with the non-CpG-added group. Data represent the mean ± standard deviation (s.d.).

CpG is unable to sufficiently up-regulate the hapten-presenting ability of purified LCs but able to enhance the LC ability in the presence of keratinocytes

EC suspensions freshly isolated from naive mice were first precultured for 24 h with various concentrations of CpG or without it as control, and were subjected to Ficoll gradient separation for LC–ECs. LCs were further purified from the LC–ECs with anti-CD11c microbeads, and 70% CD11c$^+$ I-A$^+$ cells were obtained (Fig. 3a) and modified subsequently with TNP. As responders, immune CD4$^+$ T cells were prepared from LNCs of TNP-sensitized mice. They were cultured with the TNP-haptenized LCs to examine T cell proliferation. The culture was maintained for 72 h in the presence of the same TLR ligand as that used for preincubation of ECs. As shown in Fig. 3a, the purified and haptenized LCs duly induced the proliferation of immune T cells in the absence of CpG. The addition of CpG to the LC and T cell culture slightly enhanced the T cell responses, but the percentage augmentation by CpG was as low as 67% at most. Thus, the exposure of purified LCs to TLR9 ligand did not augment LC function sufficiently.

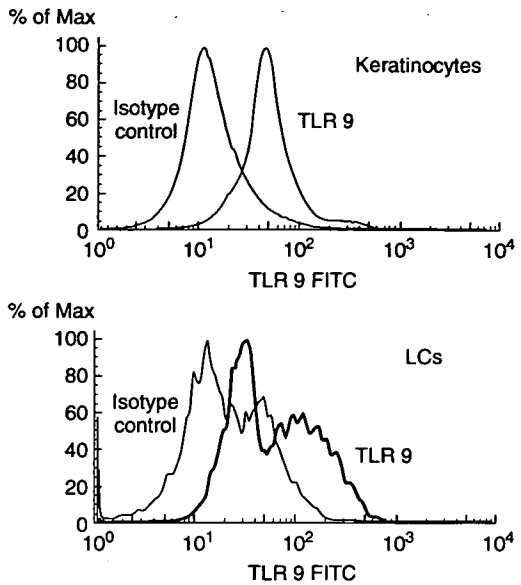

Fig. 2. Both keratinocytes and Langerhans cells (LCs) express Toll-like receptor 9 (TLR9). Epidermal cell (EC) suspensions were analysed for the expression of TLR9 by flow cytometry. For the intracellular detection of TLR9, cell fixation–permeabilization was performed before immunolabelling with anti-TLR9 and anti-I-A^d antibodies. LCs or keratinocytes were gated by I-A^d positivity.

Even if purified LCs were low-responsive to CpG, the possibility remained that their function was augmented by the ligand in the presence of keratinocytes. By using LC–EC fraction, which contains not only LCs but also a high percentage of keratinocytes, we evaluated the effect of bystander keratinocytes on the antigen-presenting LC function. LC–ECs were prepared from EC suspensions precultured for 24 h with or without CpG. LC–ECs contained MHC class II^+ $CD11c^+$ LCs typically up to 19% (Fig. 3b). They were then derivatized with TNP and cultured for 72 h with TNP-primed $CD4^+$ T cells in the presence of CpG or in its absence as control. Significant proliferative responses of immune T cells to haptenized LC–ECs were obtained in the absence of CpG (Fig. 3b). Notably, the addition of CpG dramatically enhanced the T cell response to haptenized LC–ECs. The percentage augmentation was approximately fourfold greater than that of the purified LCs. The results indicated that TLR9 ligands have the potential to augment the hapten-presenting ability of LCs when LC–ECs were used as the LC source.

CpG augments CD86 expression on LCs in the presence of keratinocytes

Freshly isolated ECs were cultured for 24 h in the presence or absence of CpG and the expression of CD86 on LCs was monitored. Figure 4 shows representative flow cytometric data, which were gated for LCs by I-A^d expression. LCs

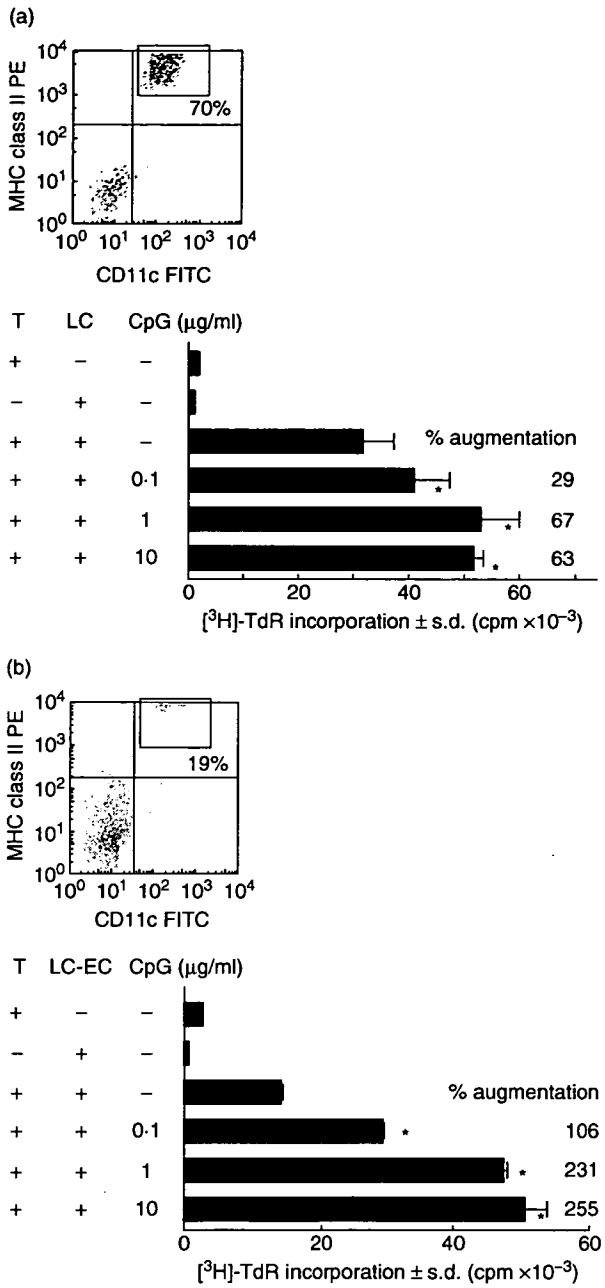

Fig. 3. CpG oligonucleotide (CpG) cannot enhance substantially the hapten-presenting ability of purified Langerhans cells (LCs), but can increase that of LC–epidermal cells (ECs). ECs were incubated with or without CpG, and LC–ECs were obtained from the ECs (b; purification of LCs, 19%). LCs were further purified from the LC–ECs positively with anti-CD11c magnetic beads (a; purification of LCs, 70%). 2,4,6-Trinitrochlorobenzene (TNCB)-immune $CD4^+$ T cells (2×10^5/well) were cultured with trinitrophenyl (TNP)-modified, purified LCs (a) or LC–ECs (b) (5×10^3 cells/well) and various concentrations of CpG. Data represent the mean ± standard deviation (s.d.). *$P < 0.05$, compared with the T + LC or LC–EC groups.

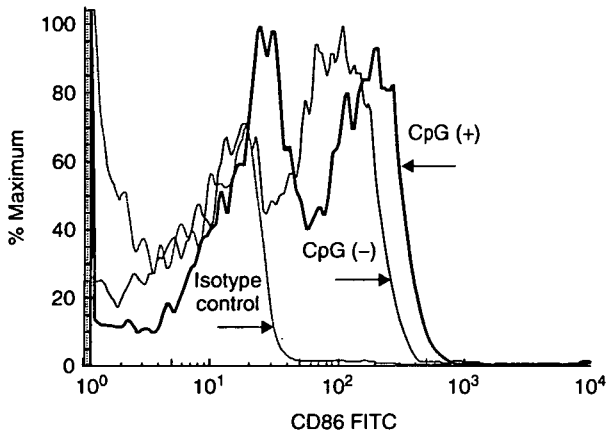

Fig. 4. CpG oligonucleotide (CpG) augments CD86 expression on Langerhans cells (LCs). Epidermal cell (EC) suspensions from naive mice were cultured with or without CpG (1 μg/ml) for 24 h. The cultured cells were subjected to flow cytometric analysis to see the expression of CD86 on LCs, which were gated by I-A^d expression. A representative flow cytometry shows the mature (CD86 high) and immature (CD86 intermediate) populations of LCs in the CpG added group (+) and the non-added one (−). The dotted line represents the isotype-matched control.

usually have two populations in their CD86 expression after 24-h culture. CpG increased the CD86-highly expressed (mature) population of LCs. Three independent series of experiments confirmed this increment of mature LCs by CpG.

CpG enhances cytokine production by keratinocytes

The above findings raised the possibility that CpG first stimulates keratinocytes to produce cytokines, thereby subsequently promoting LC function. To address this issue, keratinocytes cultured primarily from BALB/c mice were incubated for 72 h with CpG, and the culture supernatants were subjected to ELISA to quantify IL-1α, GM-CSF and TNF-α, which are keratinocyte-derived cytokines concerned with the antigen-presenting function of LCs. As shown in Fig. 5, the production levels of IL-1α, GM-CSF and TNF-α were significantly increased by CpG in a dose-dependent manner.

CpG up-regulates the antigen-presenting ability of LCs by promoting keratinocyte production of cytokines

We dissected the role of IL-1α, GM-CSF and TNF-α in the T cell response to haptenized LC–ECs under stimulation with CpG by blocking these cytokines with a cocktail of specific MoAbs. $CD4^+$ T cells were cultured with haptenized LC–ECs and CpG in the presence or absence of anti-IL-1α, anti-GM-CSF and anti-TNF-α MoAbs. Figure 6a shows that the T cell proliferation was significantly lower in the MoAb-added group than in the non-added group. Therefore, these keratinocyte-derived cytokines mediated the promoted antigen-presenting ability of LCs.

To examine the contribution of each cytokine to the antigen-presenting ability of LCs, TNP-immune T cells were cultured with TNP-modified LC–ECs and 10 μg/ml CpG in the presence or absence of two of anti-IL-1α (2·5 μg/ml), anti-GM-CSF (2·5 μg/ml) and anti-TNF-α (2·5 μg/ml) MoAbs. Percentage suppression with each combination of the two MoAbs and all the three MoAbs were as follows: anti-IL-1α + anti-GM-CSF, 23·3%; anti-IL-1α + TNF-α, 10·8%; anti-GM-CSF + anti-TNF-α, 23·2%; and anti-IL-1α + anti-GM-CSF + anti-TNF-α, 40·8%. These data suggested that all three cytokines are required for the activation of LCs and GM-CSF may be the most critical.

To further confirm the augmentative role of keratinocyte cytokines, immune $CD4^+$ T cells were cultured with anti-CD11c-purified, TNP-modified LCs in the presence of varying dilution of supernatant from CpG-stimulated keratinocytes that were used in Fig. 5. As shown in Fig. 6b, the supernatant strongly increased LC function, and thus replaced the effect of keratinocytes residing in the LC–EC fraction. These findings suggested that LC function was up-regulated indirectly by CpG via cytokines released by CpG-stimulated keratinocytes.

Discussion

It has been shown that LCs, differing from DCs, are incapable of responding to TLR ligands, as the expression of

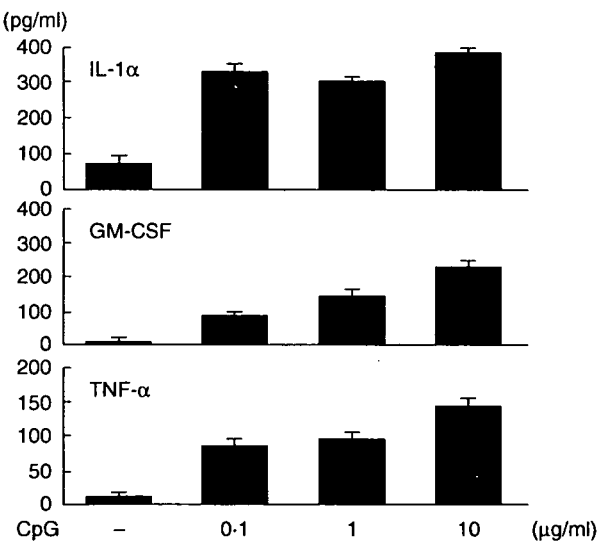

Fig. 5. CpG oligonucleotide (CpG) enhances keratinocyte production of cytokines. Freshly isolated keratinocytes were cultured for 72 h with various concentrations of CpG. Tumour necrosis factor (TNF)-α, interleukin (IL)-1α and granulocyte–macrophage colony-stimulating factor (GM-CSF) in culture supernatants were quantified by enzyme-linked immunosorbent assay (ELISA). Data represent the mean ± standard deviation (s.d.).

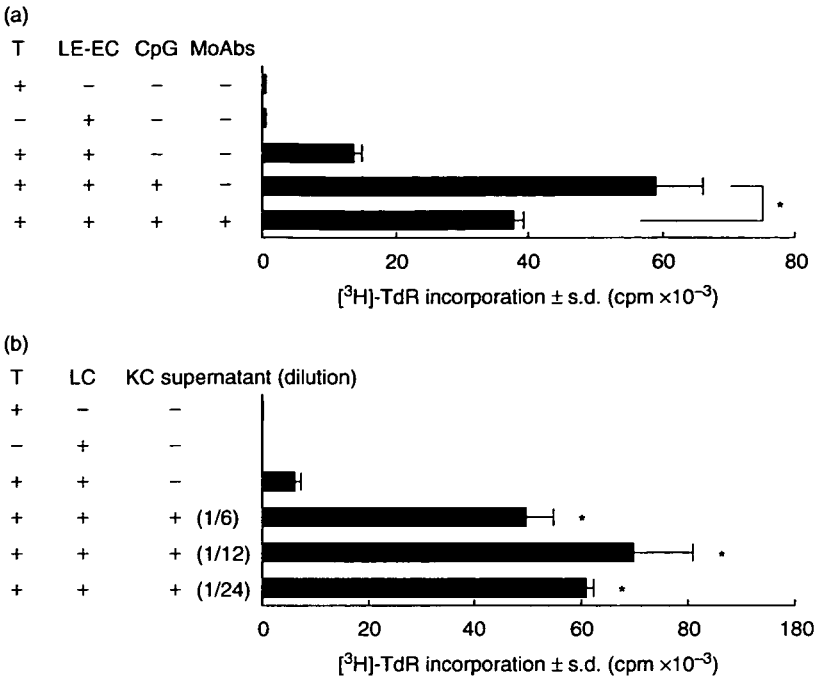

Fig. 6. Increasingly produced keratinocyte cytokines by CpG oligonucleotide (CpG) up-regulate the hapten-presenting ability of Langerhans cells (LCs). Neutralizing antibodies to keratinocyte cytokines, Interleukin (IL)-1α, granulocyte–macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor (TNF)-α, reduced the hapten-presenting ability of CpG (10 μg/ml)-stimulated LC–epidermal cells (ECs) (a). EC suspensions were incubated with CpG, and LC–ECs were prepared and haptenized with trinitrophenyl (TNP). TNP-immune $CD4^+$ T cells were cultured with TNP-modified LC–ECs in the presence of CpG. A cocktail of neutralizing monoclonal antibodies (MoAbs) to IL-1α (2·5 μg/ml), GM-CSF (2·5 μg/ml) and TNF-α (2·5-μg/ml) were added to both the starting EC suspensions and the final culture with $CD4^+$ T cells. $^*P < 0{\cdot}05$, compared with the MoAbs non-added culture. Data represent the mean ± standard deviation (s.d.). Culture supernatants from TLR ligand-stimulated keratinocytes enhanced the hapten-presenting ability of purified LCs (b). Culture supernatants from keratinocytes stimulated for 72 h with 10 μg/ml CpG were used in this study. LCs were purified positively with anti-CD11c magnetic beads. TNP-immune $CD4^+$ T cells (2×10^5/well) were cultured with TNP-modified LCs (5×10^3 cells/well) in the presence of varying dilution of keratinocytes supernatants. The numbers in parentheses indicate the final dilution. $^*P < 0{\cdot}01$, compared with T + LC group. Data represent the mean ± s.d.

MHC class II and co-stimulatory molecules on LCs are not augmented by the TLR-mediated signal [6]. Although this notion was confirmed by the present study, we also found that CpG is capable of stimulating LCs in a certain condition, resulting in enhancement of their hapten-presenting function. This stimulatory effect was exerted only when LCs co-existed with keratinocytes, as purified LCs mounted the CpG-induced enhancement at a lower level than did LC–ECs. The mediation of keratinocytes was clearly proved by the following findings: (1) CpG stimulated keratinocytes to secrete the three cytokines promoting the antigen-presenting function of LCs; (2) blocking of these cytokines with specific MoAbs abrogated the CpG-induced enhancement of LC–EC function; and (3) the addition of culture supernatant from CpG-exposed keratinocytes had the same augmentative effect as bystander keratinocytes on purified LCs. Therefore, TLR9 ligand is able to enhance LC function indirectly but substantially by promoting keratinocyte production of cytokines. It should be noted that TLR9 was expressed by normal BALB/c keratinocytes in primary culture, because this is different from the previous RT–PCR observation in murine keratinocytes [6]. However, information on the culture condition and passage of keratinocytes are missing in their report. The expression of TLR9 in normal human keratinocytes [5,13] may support our finding.

TLR ligand-enhanced production of cytokines/chemokines has been reported in several epithelial cells [14]. Intestinal and uterine epithelial cells express functional TLRs and their expression may be increased upon activation of cells [15,16]. Stimulation of these epithelial cells with TLR agonists triggers the increased expression of a number of cytokines and chemokines as seen in our study. Therefore, TLR ligands initiate innate immune responses by inducing the production of proinflammatory cytokines and chemokines as well as anti-microbial peptides, which are essential for host defence and survival. Furthermore, the present study suggests that the stimulation of keratinocytes with TLR9 ligand results in the activation of bystander LCs or DCs as a sequential event.

CpG stimulated keratinocytes to produce IL-1α, GM-CSF and TNF-α in a dose-dependent manner. The

positive feedback in the interaction between TLR expression and cytokine production has been suggested in keratinocytes [5]. Among EC-derived cytokines, GM-CSF maintains the viability and potentiates the antigen-presenting function of LCs [17]. Koch *et al.* reported that IL-1 enhances LC function twofold when combined with GM-CSF [18]. This seems to be accordance with our findings that GM-CSF neutralizing antibody is the most suppressive for the antigen-presenting activity of LCs. IL-1 further augments the T cell stimulatory activity of LCs induced by GM-CSF [19], and exposure to TNF-α enhances the viability of LCs [18]. GM-CSF may not be the predominant regulator of MHC class II and B7 expression on LCs [20], while IL-1 and TNF-α are the candidates that up-regulate MHC class II, CD54 and CD86 on LCs [21,22]. Thus, it is difficult to define actual regulators of the expression of MHC class II and co-stimulatory molecules on LCs in the epidermal milieu where many biologically active substances are released [23]. Nevertheless, it is most likely that the enhanced expression of co-stimulatory molecules on LCs observed in this study results from interactions among these cytokines.

Our finding is clinically significant. The skin is considered to be frequently exposed to TLR ligands derived from bacteria [24], or possibly viruses. Keratinocytes are the initiator to respond to TLR9 ligands and enhance the antigen-presenting function of LCs with their augmentatively produced cytokines. In parallel with this study, we also found that other TLR ligands derived from Gram-positive or negative bacteria, such as peptidoglycan and LPS, enhance the hapten-presenting ability of LCs by augmenting of keratinocyte cytokine production. Therefore, in addition to releasing anti-microbial peptides [25], keratinocytes serve as an up-regulator of acquired immunity upon exposure to microbes. Even if LCs are low-responsive to skin commensals, they can be stimulated with microbial elements indirectly via keratinocytes. On one hand, this may be a mechanism underlying exacerbation of atopic dermatitis by skin-colonized *Staphylococcus aureus* as well as the superantigen mechanism [10]. On the other hand, the subsequently activated acquired immunity may further eliminate microbes.

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Oral allergy syndrome caused by raw fish in a Japanese sushi bar worker

Contact Dermatitis 2007: 56: 369–370

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Oral allergy syndrome (OAS) is a complex of symptoms induced by exposure of the oral and pharyngeal mucosa to food allergens. A 23-year-old female presented in 2006 with oropharyngeal irritation and facial angioedema occurring within 15 min after eating raw fish. Her medical history was significant for mild atopic dermatitis and hand eczema. She had worked in a sushi bar since 2003. Clinical symptoms and positive prick-by-prick test to raw fish confirmed the diagnosis of OAS. It is likely that, our patient was sensitized by direct hand contact with raw fish.

Key words: contact urticaria; cross-reactions; occupational; oral allergy syndrome.

Case Report

A 23-year-old female with rhinitis was referred to us in 2006 for further evaluation of her immediate type of allergy to raw fish. Her past history included mild atopic dermatitis and hand eczema in childhood. The patient had worked in a sushi bar since 2003. In 2004, when she ate sea bream, she first noticed discomfort sensation of her oral cavity. Thereafter, she had similar episodes to conger and eel in the same year, and to sardine and horse mackerel in 2005. Oropharyngeal irritation and facial angioedema occurred within 15 min after eating raw fish. In addition, she gradually developed an urticarial eruption, which was occasionally associated with diarrhoea or asthma. Since then, she had strictly avoided eating these raw fish. No ordinary contact urticaria to fish or anaphylactic shock was noticed.

On examination, the peripheral blood sample showed normal counts of leukocytes (4800/μl; normal, 3100–9700/μl) and eosinophils (144/μl, 3%; normal, 0.1–6.7%). Total immunoglobulin E (IgE) level was slightly high (200 U/ml; normal, <170 U/ml) but specific IgE antibodies against tuna, salmon, mackerel, cuttlefish, horse mackerel, and sardine were all negative. We performed prick-by-prick test with raw fish for sushi. Among them salmon (3 mm), flounder (10 mm), conger (6 mm), sea bream (5 mm), amberjack (3 mm), and tuna (7 mm) yielded positive results (> 1/2 wheal diameter of histamine). Therefore, we diagnosed the patient as having oral allergy syndrome (OAS) caused by raw fish. After avoiding eating raw fish, she got well and did not have any symptom of OAS.

Discussion

OAS is a clinical entity that remains unfully defined but its clinical importance has been rapidly increasing. Latex-fruit syndrome is the prototype of OAS, and sensitization with Latex leads to oral allergy to fruits (1). Because the oral cavity and intestinal tract are tolerance-inducible sites when exposed to external allergens, sensitization should be achieved on other sites such as skin and conjunctiva in OAS. OAS occurs within minutes of ingestion and presents as burning, swelling and itching of lips, tongue, mouth, or throat. Other symptoms that are only rarely seen include rhinoconjunctivitis, angioedema, asthma, anaphylactic shock, and their combination (2).

Our patient had oral and pharyngeal symptoms to a number of raw fish with positive prick-by-prick tests to those fish. In contrast to food-dependent allergic reactions induced via gastric or intestinal absorption, where digestion may promote the antigenicity of foods, OAS is most likely induced by exposure to native non-modified allergens (3).

The major skin manifestations evoked by occupational exposure to seafood are eczematous contact dermatitis and contact urticaria, mostly located on the hands and forearms (4, 5). Our patient was probably sensitized with raw fish during working in a sushi bar. Ordinarily, OAS occurs as a result of cross-reactivity with other contactants or pollen such as rubber latex (2). Because sensitization does not successfully occur via oral mucosa, the patient seems to have been sensitized by direct hand contact with raw fish. When she came to our hospital, her hands were intact but the atopic past history and potential skin barrier disruption of her hands support this speculation.

As has been reported in fish allergy (6), she showed broad antigenic cross-reactivity among fish. She worked in a Japanese sushi bar and was exposed to many types of fish. The possibility remains that she was sensitized with other fish that did not appear in the present history. It has been suggested that parvalbumin is an antigenic molecule for fish allergy (7). This common substance might be an allergen in our patient. She exhibited positive prick test results for other types of fish than those to which she was exposed, supporting cross-reactivity and providing beneficial information on avoidance. It should be kept in mind that raw fish can be one of the causative foods for OAS.

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