3. Results

3.1. The role of LpK lipidation in IL-12 production

To verify the role of lipid modification of LpK in terms of IL-12 production from human monocytes, various forms of truncated LpK were produced in *E. coli*. LpK-a is a lipidated protein, while other truncated proteins (LpK-t, LpK-b, LpK-d, and LpK-e) are non-lipidated (Fig. 1). The cytokine producing ability of mature lipidated LpK was first compared with non-lipidated LpK-t having the whole conserved amino acid residues of LpK. The expression vector for LpK-t was constructed by eliminating the nucleic acids coding for the N-terminal hydrophobic region up to the cysteine residue which is acylated in LpK. While 2.5 nM LpK produced more than 1000 pg/ml IL-12 p40 in human monocytes, 2.5 nM LpK-t produced IL-12 more than 9-fold less efficiently, and it required more than 10 nM to produce an equivalent dose of the cytokine (Fig. 2). This result indicated that the presence of the N-terminal lipid entity significantly enhanced the IL-12 producing activity of LpK protein from monocytes. To confirm this point, we examined the IL-12 inducing ability of another truncated protein, LpK-a. The expression plasmid encoding *lpk-a* was constructed by taking the N-terminal 82 amino acid coding nucleic acids of *lpk* including the signal peptide region. LpK-b having no lipid residue, but only the amino acid residues of LpK-a, was also produced. The uptake of radio-labeled glycerol was used to confirm the presence of lipid modification. *E. coli* expressing LpK-a was co-cultured for 5 h in the presence of [^{14}C]glycerol and the cells were lysed. When the protein was run on an SDS–polyacrylamide gel, autoradiography showed a 12 kDa radio-labeled band, which corresponded to the predicted molecular mass (not shown). The expression of the protein at the same

Fig. 3. Among the truncated proteins, LpK-a was the most efficient in inducing IL-12 from monocytes. Various truncated forms of LpK were expressed in *E. coli* and purified, then evaluated for their IL-12 inducing ability. Among LpK-a, -b, -d, -e, and lipidated LpK-a was the most efficient in inducing IL-12, but LpK-e did not induce cytokine production. The results were obtained from one experiment, but were consistent with three different experiments. Mean ± standard deviation of a triplicate assay is shown. By Student's *t* test, the following *p* values were obtained: $p < 0.01$ for values between LpK-a and LpK-b, LpK-a and LpK-e; $p < 0.005$ for values between LpK-a and LpK-d, LpK-b, and LpK-e; $p < 0.001$ for values between LpK-b and LpK-d; and $p < 0.05$ for values between LpK-d and LpK-e at 5.0 nM.

position was confirmed by Western blotting. Such a radio-labeled band was not observed in the case of LpK-b, which indicated that LpK-a was lipidated.

Next, we determined the ability of LpK-a to produce IL-12 in monocytes, and compared it with that of non-lipidated LpK-b. LpK-a was significantly more efficient at cytokine production than LpK-b (Fig. 3). While 0.5 nM LpK-a produced more than 700 pg/ml IL-12, non-lipidated LpK-b required more than 20 nM of protein to produce an equivalent dose of IL-12. One possible reason for the less efficient cytokine production by LpK-b is that it lacks some immuno-stimulatory domain, which may be the acylated structure itself or its contribution to the conformation of the protein. To further analyze the effect of other truncated LpK, we produced non-lipidated LpK protein, LpK-d, and LpK-e (Fig. 1). LpK-c, a lipidated protein having the N-terminal half of the LpK protein could not be expressed in *E. coli* for unknown reasons. LpK-d covers the N-terminal 158 amino acid residues of the LpK protein and LpK-e has amino acid corresponding to the C-terminal half of the LpK protein with a single overlapping amino acid with LpK-d. However, LpK-d induced IL-12 less efficiently compared to that produced by LpK-a. The IL-12 producing activity of LpK-d was comparable to that of LpK-b, but LpK-e had no such IL-12 stimulating ability (Fig. 3). These results may indicate that acylated N-terminal 60 amino acids of LpK is responsible for the strong immuno-stimulatory activity of LpK.

3.2. Immuno-stimulatory activity of synthetic lipopeptide

As shown in Fig. 3, the ability of LpK to induce IL-12 production in monocytes resides in the N-terminal
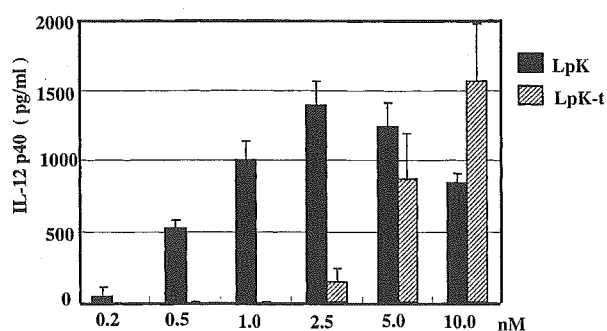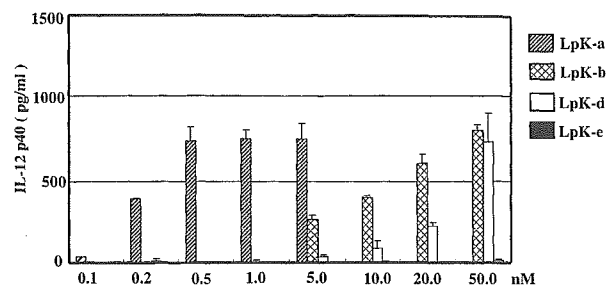

Fig. 2. LpK induces significant IL-12 production from human blood monocytes when compared to truncated LpK-t. Monocytes were isolated from healthy human blood cells as described in Section 2.4. IL-12 p40 production was measured by ELISA. The results shown are obtained from one experiment, but were consistent with three different experiments. Mean ± standard deviation of a triplicate assay is shown. By Student's *t* test, the *p* values obtained were: $p < 0.005$ for values between LpK and LpK-t at 2.5 nM and $p < 0.05$ at 5.0 nM.

region of the protein, including the acylated portion. Therefore, we synthesized lipopeptide with N-terminal 12 amino acid residues of LpK, having the N-terminal cysteine residue conjugated to palmitoylated triacylated glycerol, which is presumed to be having the same lipid composition as that of LpK purified from *E. coli*. Fig. 4 represents the IL-12 production from monocytes by synthetic LpK lipopeptide and non-lipidated peptide having the same N-terminal 12 amino acids of the lipopeptide. It was surprising to note that, 50 nM synthetic lipopeptide produced less than 200 pg/ml IL-12. Further increasing the concentration of lipopeptide, did not result in further elevation of the level of cytokine production (not shown). The synthetic non-lipidated peptide having the same amino acids as that of the lipopeptide almost totally lacked the ability to induce IL-12 production in monocytes (Fig. 4). These results suggested that lipidation of the N-terminal peptide was necessary, but was not as efficient as LpK or LpK-a, for the production of IL-12.

3.3. Association of LpK protein and TLR-2

We examined whether NF-kB-driven luciferase activity was upregulated in TLR2 transfected HEK 293 cells by LpK and its truncated protein. PG, a well-defined TLR-2-associated Ag, was used as a positive control. Significantly higher luciferase activity was observed when the TLR-2 transfected HEK293 cells were stimulated with PG or lipidated lipoprotein including LpK and LpK-a, but not in mock transfected HEK293 cells (Fig. 5). On the contrary, no significant or no antigen-dose-dependent luciferase activity was induced by any of the non-lipidated proteins such as LpK-b, LpK-d, and LpK-e. But, significant activity was observed in cells when stimulated with synthetic lipopeptide LpK, despite lacking the ability to induce IL-12 efficiently. These results suggested that TLR-2 stimulation of LpK protein is essential, but not adequate for efficient production of the cytokine.

Fig. 5. Association of TLR2 and truncated LpK proteins. NF-kB-dependent reporter gene activity of the TLR2 transfectant was measured after stimulation with LpK or its truncated forms as described in Section 2.5. Peptidoglycan (PG) was used as a positive control for TLR2 dependent luciferase activity. Data are expressed as fold induction relative to the activity of *Renilla* luciferase, which is an internal control for transfection efficiency in the Dual luciferase reporter assay. The result shown is representative of three different experiments. Assays were done in triplicate and the mean ± standard deviation is shown.

3.4. The role of the non-lipidated protein component of LpK in IL-12 production

To confirm that the stimulation of monocytes with non-lipidated components of LpK protein is required for efficient IL-12 production, we stimulated monocytes with lipopeptide in the presence of various concentrations of non-lipidated proteins (Fig. 6). When monocytes were co-stimulated with lipopeptide and LpK-b or LpK-d, they produced IL-12 in a manner dependent on the concentration of truncated non-lipidated LpK proteins, although induction of IL-12 by LpK-b was a little lower in this set of experiments due to donor variations. However, the combination of lipopeptide and LpK-e or synthetic peptide did not induce any cytokine production. These results suggested that lipopeptide by itself is ineffective in producing IL-12, but is markedly synergistic with certain immuno-dominant regions of LpK, which apparently seem to correspond to the N-terminal 60 amino acid residues.

4. Discussion

The clinical manifestations of leprosy appear based on the immunological spectrum according to the level of cell-mediated immunity to *M. leprae*. Lepromatous leprosy patients manifest disseminated infection, their T cells respond weakly to the bacilli and their lesions express type 2 cytokines. In contrast, tuberculoid patients mount a strong Th1 response to *M. leprae*. When a Th1 cell-mediated immune response is generated, clinically apparent leprosy infection is localized, leading to the formation of a granuloma. For efficient induction of Th1 response, IL-12 is envisaged to play an
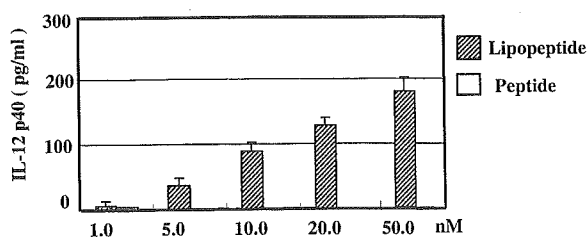

Fig. 4. The synthetic lipopeptide derived from the sequence of N-terminal LpK showed ability to induce IL-12, but to a limited extent. The same peptide sequence as that of the lipopeptide sequence without the acyl attachment did not induce any cytokine production. A representative of three independent experiments is shown. Each experiment was performed in triplicate and the mean ± standard deviation is shown. By Student's *t* test, the *p* values obtained were: $p < 0.05$ for values between lipopeptide and peptide at 5.0 nM.
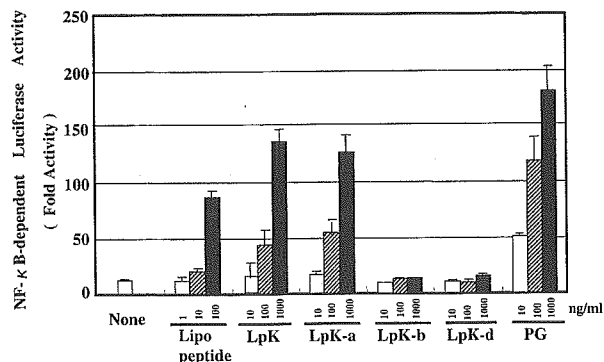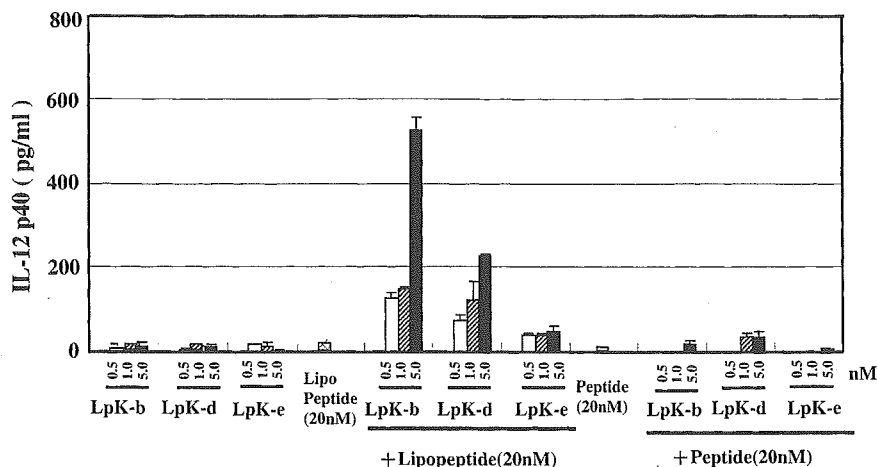
Fig. 6. The presence of lipopeptide enhances the IL-12-inducing ability of non-lipidated truncated proteins of LpK, namely Lpk-b and LpK-d, but not that of LpK-e. Values expressed are the mean values ± SD of triplicate samples and are representative of three independent experiments.

important role. Presently, a number of studies are being conducted to precisely evaluate the relationship between IL-12 and mycobacterial diseases [15–20]. Early leprosy progression is controlled by type 1 cytokines, including IL-12, as observed in single-skin lesion tuberculoid leprosy, and resistance to experimental *M. leprae* infection has been correlated with the early production of IL-12 at the site of infection [21,22]. Also, active tuberculosis is associated with reduced type 1 responses to *M. tuberculosis*, the response of which is efficiently induced by IL-12. Furthermore, human patients with inherited IL-12 or IL-12-receptor deficiency showed higher susceptibility to even primary mycobacterial infection, including environmental and less pathogenic mycobacteria such as *Mycobacterium bovis* bacillus Calmette Guérin [23]. These data indicate that IL-12 is closely associated with the activation of protective immunity against mycobacterial infection.

IL-12 is chiefly produced from APCs such as monocyte/macrophages and dendritic cells (DCs), which are the most susceptible cells in vivo to *M. tuberculosis* and *M. leprae* infection. The activation of APCs takes place by the engagement of antigenic molecules with the ligands ubiquitously expressed on the cell surface, and is closely associated with innate immunity. Therefore, molecules capable of inducing IL-12 production from monocytes largely contribute to evoke anti-mycobacterial host defense activities. Recently, we isolated an *M. leprae* lipoprotein, LpK, and indicated that LpK stimulated monocytes to produce IL-12 [6]. Although the receptor molecules for LpK are unknown, TLR-2 is reported to be involved in interactions with mycobacterial lipoprotein such as 19-kDa *M. tuberculosis*-derived lipoprotein, and PG [3,24,25]. In the present study, we produced various form of truncated LpK and analyzed the role of TLR-2 and lipidated or non-lipidated proteins in the production of IL-12 from monocytes, since

monocytes are the APCs that first encounter *M. leprae* in vivo.

NF-kB-driven luciferase activity in TLR-2-transfected HEK 293 cells was upregulated when stimulated with LpK, as well as the lipid modified truncated LpK-a and lipopeptide, while the activity was not seen when stimulated with the non-lipidated LpK proteins. However, it was observed that non-lipidated LpK-t and LpK-b, in addition to lipidated forms of LpK, efficiently induced IL-12 production in monocytes. Although there are reports suggesting that heat shock protein 70, which is a non-lipidated protein, utilizes TLR-2 to transduce its proinflammatory signal [26], the lack of activation by non-lipidated LpK protein, especially LpK-t, indicates the absence of TLR-2 involvement in the non-lipidated LpK region for efficient IL-12 production. However, the significant production of IL-12 in monocytes was not induced by sole ligation of TLR-2 with a lipid component, as indicated by the lack of efficient IL-12 production by a synthetic LpK lipopeptide. This observation might be on line with the findings that glycolipids do not induce cytokine production from monocytes. But inflammatory cytokine TNF-α, which is also induced via ligation to TLR2 [27], has been efficiently induced by the lipopeptide (data not shown). Therefore, it can be emphasized that additional stimulation by the polypeptide region of LpK through some unidentified receptors is required for the efficient production of IL-12 from monocytes. When higher concentrations of non-lipidated LpK proteins such as LpK-t and LpK-b, were pulsed to monocytes, they produced comparable level of IL-12 to that produced by 1–2 nM of lipidated LpK. This implies that the protein component by itself can activate monocytes. Recently, a number of receptor molecules on macrophages and DCs, such as mannose receptors, complement receptors, DC-SIGN, and CD14 molecules, have been extensively analyzed, and

have been shown to be capable of binding mycobacterial components, while the receptors associated with IL-12 production from monocytes have not been fully elucidated. However, the results suggest that the non-lipidated protein region of LpK is involved in IL-12 production in monocytes and we are now examining the host surface molecules involved in polypeptide binding for efficient IL-12 production.

From the aspect of IL-12 production, the N-terminal portion of *M. leprae*-LpK is important since LpK-a carrying both a lipidated and hydrophobic protein region stimulated IL-12 as efficiently as parent LpK. Therefore, LpK-a, for which lipidation is confirmed by integration of radio-labeled glycerol into the protein, can be a substitute for LpK. In addition to innate immunity, adaptive immunity such as IFN-γ production from type 1 CD4$^+$ T cells also plays an important role in the protective immunity against mycobacterial infection [28–30]. Although IL-12 does not directly act as an initiator of adaptive immunity, it can work as a bridge between innate and adaptive immunity [9,31]. We are now evaluating the antigenicity of LpK and LpK-a in DC-mediated, MHC or CD1 molecule-restricted cellular immunity in vitro and also underway to determine the efficacy of LpK in vaccine development by conducting animal experiments.

In conclusion, *M. leprae* lipoprotein produced IL-12 in monocytes by stimulating them through two or more signaling pathways, and both components, the triacylated lipid region and the polypeptide region were required for efficient IL-12 production. Therefore, the potential contribution of LpK to protective immunity against *M. leprae* infection is highly anticipated.

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HLA-mediated signaling via HLA-peptide-TCR complex determines immune responses of antigen-presenting cells

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ABSTRACT

When HLA-DR, -DQ and -DP molecules were cross-linked by solid-phase mAbs, monocytes produced monokines, and only anti-DR Ab markedly activates MAP kinase Erk, whereas anti-DR, anti-DQ and anti-DP all activate MAP kinase p38. DR-restricted T cells that are established from PBMC and are reactive with mite antigens, PPD and random 19-mer peptides, exhibited higher IFN-γ : IL-4 ratio than did DQ- or DP-restricted T cells. These results indicate that HLA-DR, -DQ and -DP molecules transmit distinct signals to monocytes via MAP kinases and lead to distinct monokine activation patterns, which may affect T-cell responses *in vivo*. Thus, the need for generation of a multigene family of class II MHC seems apparent. HLA-DR on B cells, on the other hand, not only present antigenic peptides to T cells, but also up-regulate IgM production, in association with Syk activation. When HLA-DR or CD3 molecules on cloned CD4$^+$ T cells were cross-linked by solid-phase mAbs, T cells proliferated, and this resulted in anergy. We propose that signaling via HLA-DR molecules on CD4$^+$ T cells at least in part contributes to the induction of T cell anergy that can be induced by soluble form of antigenic peptide. We next used IFN-γ-treated and irradiated periodontal ligament fibroblasts (PDL) expressing HLA-DR molecules. Indeed, Th cells did not show proliferative responses

when peptide-pulsed PDL were used as APC, whereas PDL produced larger amounts of IL-6, IL-8, MCP-1 and RANTES compared with controls, when cultured with anti-HLA-DR mAb or emetine-treated T cells. These findings suggest that HLA-DR expressed on fibroblasts may act as receptor molecules that transmit signals into fibroblasts, based on DR-peptide-TCR interaction, resulting in the secretion of several cytokine species.

INTRODUCTION

We earlier reported that interactions between a CD4$^+$ T cell clone and monocyte via altered TCR ligands, affect monocyte responses to produce IL-12 with marginal involvement of CD40, events which lead to specific up-regulation of IFN-γ production from T cells (1). Thus, signals transmitted to monocytes via class II HLA molecules are involved in determining immune response patterns. It is highly conceivable that signals transmitted by class II MHC molecules in B cells, in regulating APC function during cognate T-B-cell interactions, are important, for the following reasons: (*a*) cross-linking class II molecules induces an increase in intracellular calcium and cAMP in mouse or human B cell lines (2-5); (*b*) class II MHC-mediated signals lead to homotypic aggregation of B cells (6); (*c*) cross-linking HLA-DR molecules on B cells induces apoptosis (7); (*d*) class II MHC molecules

without the intracellular domain expressed on B lymphoma cells will not lead to an increase in cAMP and subsequent CD80 up-regulation, when stimulated with a CD28-expressing autoreactive T hybridoma cells (8); (*e*) cytoplasmic domain mutants of class II MHC abrogate generation of intracellular cAMP (9) and translocation of PKC (10); and (*f*) cross-linking HLA-DR molecules expressed on B cells induces phosphorylation of Src family kinases (Lyn, Fgr) (11) and Syk (12). Moreover, engagement of class II molecules on the THP-1 monocyte cell line with Staphylococcal enterotoxin A induced IL-1β and TNF-α (13). While functional consequences of such DR-mediated signaling events induced by T cells are largely unknown, these observations do raise the possibility that signaling through class II MHC molecules may affect monocyte responses as well, including monokine secretion, upon TCR-TCR ligand interaction.

Our previous investigations on HLA-DR vs -DQ (14) or on I-A vs I-E by others (15) suggested their distinct roles in activating Th / Ts. Thus, HLA-DR function as an Ir-gene for shistosomal antigen-specific immune responses, whereas HLA-DQ do as an Is-gene, being epistatic to DR. However, their roles in activating Th1 / Th2 have remained elusive. To investigate the consequence of signaling events through distinct subregion products of class II HLA, we first tested monokine secretion patterns induced by (*a*) solid-phase mAbs to HLA-DR, -DQ and -DP molecules expressed on peripheral blood adherent monocytes, and (*b*) co-culture of peptide-pulsed monocytes with emetine-treated T cell clones of various HLA restriction patterns (16).

MATERIALS AND METHODS

Reagents

Anti-HLA class II mAb HU4 (anti-HLA-DRB1+DRB5 IgG2a, monomorphic), L243 (anti-HLA-DRB1+DRB4 IgG2a, monomorphic), HU11 (anti-HLA-DQ4+5+6 IgG2a), HU18 (anti-HLA-DQ7+8+9 IgG2a) or B7/21 (anti-HLA-DP IgG1, monomorphic) (1) were as described. Anti-HLA class II mAbs 1a3

(anti-HLA-DQ IgG2a, monomorphic) (Leinco Technologies, Inc. Manchester, UK) were purchased. Mouse IgG, IgG1, and IgG2a were purchased for control, from BioPur AG (Bubendorf, Switzerland) and Biogenesis (Poole, UK). Abs were purified from the ascites-form of mAbs, using a Protein-A column (PIERCE, IL). F(ab')2 fragments of L243 and mouse IgG were prepared, using ImmunoPure F(ab')2 Preparation Kits (Pierce) with extensive dialysis to remove residual Fc fragments. Genistein (Sigma, St. Louis, LA), GF109203X (Sigma), piceatannol (Sigma), PD98059 (New England Biolabs, Beverly, MA) and SB203580 (Calbiochem, La Jolla, CA) were purchased. *Dermatophagoides farinae (Der f)* antigens were kindly provided by Torii Pharmaceuticals (Tokyo, Japan). PPD was purchased from Japan BCG Laboratory (Tokyo, Japan). Peptides with defined sequences were synthesized using a solid-phase simultaneous multiple peptide synthesizer PSSM-8 (Shimadzu Corp., Kyoto, Japan) based on the Fmoc strategy and using a ten-fold molar excess of single Fmoc-amino acids, then were purified using C18 reverse-phase high-performance liquid chromatography. In the case of degenerate peptides, the introduction of randomized sequence positions was done in a double coupling step with equimolar mixtures of Fmoc-L-amino acids, used in an equimolar ratio with respect to coupling sites of the resins (all positions have 19 amino acid residues except Cys).

Human T cell clones

Human CD4+ T cell clone BC20.7 that recognizes DR14 (DRA + DRB1*1405) + residues 84-100 of BCGa protein (BCGap84-100; EEYLILSARDVLAVVSK), has been described previously (17). OT1.1 (18) and DT13.2 (1) are specific for DP5 (DPA1*0201 + DPB1*0501) + p53p153-165 (STPPPGTRVRAMAIYKQS) and DQ6 (DQA1*0102 + DQB1*0602) + *Der f* Ip18-31 (RSLRTVTPIRMQGG), respectively. T cell clones were fed weekly with 50 U/ml human rIL-2 and 10 U/ml human rIL-4, in the presence of irradiated autologous PBMC prepulsed with each peptide, in RPMI 1640

medium (Gibco, Grand Island, NY) supplemented with 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% pooled, heat-inactivated normal human male plasma in 24-well flat-bottomed culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ). Culture medium and Ab preparations tested for contamination with endotoxin, exhibited negative results. Human bleeding and animal experiments (ascites preparation) were in accordance with institutional guidelines.

Preparation of adherent APC

PBMC were freshly prepared from heparinized blood of healthy adult donors, using Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ). The PBMC were incubated at 3×10^7 cells in 10 ml of 10% HS/RPMI for 1.5 h in 90-mm culture-grade plastic petri dishes pre-coated with heat-inactivated autologous plasma, at 37°C in a CO2 incubator. After removing non-adherent cells, the adherent cells were recovered from plates by incubating with ice-cold 0.05% EDTA/PBS for 10 min and repeated pipeting. Monocytes were cultured for 48 h to allow adherence-induced transcription of monokine mRNA to subside (19). This population was composed principally of monocytes and were > 90% CD14-positive, as analyzed by FACS (not shown). *HLA class II (DR, DQ and DP)* alleles were determined, as described elsewhere (1). HLA type of the two monocyte donors were DRB1*0101/1201 and DRB1*1405/1502, both of which are negative for DRB4.

Stimulation of monocytes

Ten μg/ml anti-DR Ab (L243), anti-DQ Ab (1a3), anti-DP Ab (B7/21), mouse IgG (alternatively, IgG1 and IgG2a) were pre-coated onto 96-well flat-bottomed culture plates. Adherent cells were incubated at 6×10^4 cells/well where mAbs are immobilized, at 37°C in a CO2 incubator. Culture supernatants were collected at 6, 16, 24, 48 and 72 h and stored in aliquots at -80°C until determinations of lymphokine concentrations.

Alternatively, T cells treated with 0, 10, 30 and 90 μg/ml of *de novo* protein synthesis

inhibitor emetine (Sigma) (20) for 1 h at 37°C were washed three times with RPMI 1640 medium. Cells were re-suspended in culture medium, incubated for 3 h at 37°C, then washed three times with RPMI 1640 medium and co-cultured with peptide-pulsed or mock-pulsed monocytes. Culture supernatants after 16- (for IL-12), 24- (for IL-1β, IL-10, IL-18, GM-CSF and TNF-α) and 48-h (for IL-6) incubation were collected, and subjected to ELISA. Treatment of T cells by emetine abrogated IL-4 production from BC20.7 (BCGa-specific, DR14-restricted), in a dose-dependent manner; ninety μg/ml of emetine treatment resulted in a complete abrogation of IL-4 production, but not IL-12 produced by peptide-pulsed monocytes (not shown). Moreover, culture supernatants of the peptide-pulsed monocytes stimulated with emetine-treated T cells were positive for IL-12 production, but not so mock-pulsed monocytes stimulated with emetine-treated T cells (not shown). The interaction between HLA and peptide alone did not induce monokine production. Results were similar in case of HLA-DQ-restricted DT13.2 and HLA-DP-restricted OT1.1 (not shown).

ELISA assays

The human IL-4, IFN-γ, IL-1β, 10, 12 (p40 + p70), GM-CSF and TNF-α ELISA kits (Biosource International) and human IL-6 ELISA kit (Genzyme) were used for quantitation of lymphokines in the supernatants, according to manufacturer's instructions. ELISA kit for IL-18 was kindly provided by Dr. M. Kurimoto (Hayashibara Biochemical Laboratories, inc., Okayama, Japan). Statistical significance was analyzed using Student's t test.

Western immunoblot analysis

Monocytes prepared from PBMC were added to 96-well culture plates in which class II HLA mAbs had been immobilized, followed by centrifugation. After 10-60-min incubation at 37°C, ice-cold 100 μM sodium vanadate/PBS was added for washing, followed by lysing in 50 μl of lysing buffer (150 mM NaCl, 20 mM Tris, pH7.6, 0.5% Nonidet P-40, 2 mM Na-orthovanadate, 1 mM NaF, 5 mM EDTA plus a protease inhibitor cocktail purchased from

SIGMA). After centrifugation, supernatant fluids of the lysates were electrophoresed on SDS-PAGE gels and transferred to nitrocellulose membrane. After blocking with 10% skim milk, 0.2% Tween-20 in Tris-buffered saline, the membrane was incubated with Abs specific for Erk, JNK and p38 (Santa Cruz) or with Abs specific for the activated form of Erk, JNK and p38 (Upstate Biotechnology, NY), washed extensively and subjected to chemiluminescence detection with peroxidase-conjugated anti-mouse IgG Ab, using an ECL kit (Amersham, U.K.). Signals were analyzed using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov.).

Establishment and analysis of Der f-, PPD- and X19-reactive T cell lines

Der f (crude mite antigen)-specific short-term T cell lines were established from PBMC from two donors carrying different HLA types (MA: HLA-DRB1*1405 / DRB1*1502, NI: HLA-DRB1*0901 / DRB1*1302). HLA-DR-restricted and HLA-DP-restricted T cell lines were established by co-culture either with anti-HLA-DQ (HU11 and/or HU18) + anti-HLA-DP (B7/21) mAbs or with anti-HLA-DR (HU4 and L243) + anti-HLA-DQ (HU11 and/or HU18) mAbs, respectively, in the presence of the crude extract of *Der f*. Restriction molecules of these cell lines were confirmed by inhibition assays with mAbs (not shown), and all the cell lines of expected restriction patterns were used for the analysis. These cell lines were restimulated with excess concentrations of antigens (10 μg/ml), then after 48-h incubation, culture supernatants were collected for measurements of IFN-γ and IL-4 production by ELISA. PPD-specific short-term T cell lines were established from PBMC of donor MA. HLA-DR-restricted and HLA-DQ-restricted T cell lines were established by co-culture with anti-HLA-DQ (HU11) + anti-HLA-DP (B7/21) mAbs or anti-HLA-DR (HU4 and L243) + anti-HLA-DP (B7/21) mAbs, respectively, in the presence of PPD. X19 (19-mer peptides with random sequences)-reactive T cell clones were

established from PBMC of donor MA, using X19, IL-4, IL-7, IL-9, IL-15 and agonistic Ab to CD29, under cloned. Restriction molecules were determined by inhibition assays with mAbs.

In vitro immune-complex kinase assay

The human B lymphoblastoid cell line LD2B (1×10^7) was incubated for 10 min on ice and then pre-incubated either with biotinylated Igs (40 μg / 200 μl) or with biotinylated F(ab')2 fragments (12 μg / 200 μl) for 10 min on ice. After washing with ice-cold RPMI1640, the cells were suspended with 50 μl of 10 % FCS / RPMI and cross-linked with 50 μl of avidin (1 mg / ml). After 10-min incubation at 37°C, ice-cold 100 μM Na3VO4 / PBS was added, followed by pelleting and lysing in 400 μl of the lysing buffer. Supernatant fluids of the lysates were pre-cleared with Protein A-agarose beads, then were incubated with a rabbit polyclonal anti-Syk Ab (Santa Cruz Biotechnology, Inc.), using Protein A-agarose beads (PIERCE). After shaking for 30-min at 4°C, the beads were washed 4 times with lysis buffer. An aliquot of immunoprecipitated proteins was eluted with Laemmli buffer containing 2-ME, for immunoblotting analysis. Residual beads were washed once with kinase buffer (25 mM HEPES [pH7.4], 0.1%[v/v] Nonidet P-40, 10 mM MgCl2, 3 mM MnCl2, 30 μM Na3VO4; 30) and were re-suspended in 30 μl of the kinase buffer containing 2 μg (0.11 nmol) MBP (SIGMA), in the presence of either 27.5 nmol HS1 peptide or an irrelevant peptide. Reactions were initiated by adding 3.75 μM [γ-^{32}P]ATP (10 μCi of [γ-^{32}P]ATP/sample, 5000 Ci/mmol; Amersham), then incubated for 2.5 min at 25 °C. The reactions were terminated by adding an equal volume of 2x Laemmli buffer. The supernatants were boiled for 2 min and applied to a 12% SDS-PAGE. After electrophoresis, the gel was fixed and vacuum dried, and analyzed using a bio-imaging analyzer (BAS2000, Fuji Film, Tokyo). Eluted protein samples were separated on 7.5 % SDS-PAGE and transferred to nitrocellulose membrane. After blocking with 10 % skim milk, 0.2 %

Tween-20 in Tris-buffered saline, the membrane was incubated with the rabbit anti-Syk Ab, washed extensively and subjected to chemiluminescence detection with peroxidase-conjugated anti-rabbit IgG Ab (Santa Cruz Biotechnology, Inc.), using an ECL kit (Amersham).

Anergy induction assay

SF36.16 T cells were primarily stimulated with soluble-form wild-type peptide (1 µM BCGap84-100), immobilized anti-DR mAb, immobilized anti-CD3 mAb or irradiated autologous PBMC prepulsed with the wild-type peptide (5µM BCGap84-100 for 5h at 37 °C) in 24-well flat-bottom culture plates. Seven days later, these T cells were washed with culture medium and co-cultured with irradiated autologous PBMC in the presence of various concentrations of wild-type BCGap84-100. The T cells were cultured in a 96-well plate for 72 h, and subjected to [³H]thymidine incorporation assay.

Fibroblasts

The human PDL used in this study was isolated from two periodontally healthy donors carrying DRB1*1501-DQB1*0602-DPB1*0501 / DRB1*0405-DQB1*0401-DPB1*1901, and DRB1*1302-DQB1*0604-DPB1*0301 / DRB1*0901-DQB1*0303-DPB1*0401 haplotypes. PDL were maintained in a medium consisting of Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS; Irvine Scientific, Santa Ana, CA, USA), 2 mM glutamine, 50 µg/ml gentamicin, 0.2 mM non-essential amino acids (all additives were from Life Technologies) at 37°C in 95% air and 5% CO_2. All experiments were carried out while these cells were actively growing between passages 3-8. The expression of HLA-II molecules on fibroblasts treated with or without IFN-γ was evaluated using flow cytometry with Epics (Beckman Coulter, Fullerton, CA).

RESULTS

Monokine production induced by anti-HLA mAbs

We examined the monokine secretion induced by cross-linking class II HLA molecules, using solid-phase mAbs to class II HLA, by which involvement of cell-surface molecules other than HLA is unlikely to occur. As shown in Fig. 1A, the effect of the anti-DQ
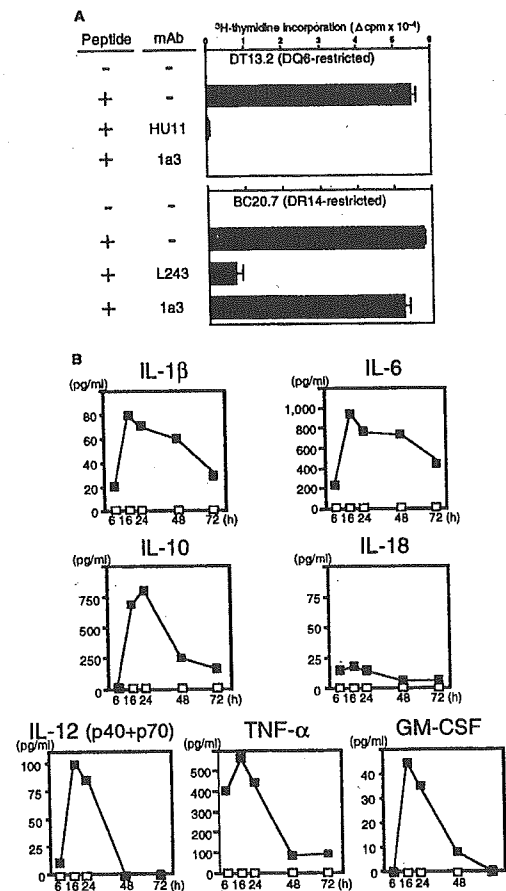
Figure 1. Monokine secretion induced by solid-phase mAbs to HLA. (A) DT13.2 and BC20.7 were cultured in the presence of Der f 1 peptide (for DT13.2) or BCGa peptide (for BC20.7) and irradiated autologous PBMC, with or without anti-class II HLA mAbs. (B) Adherent cells were incubated at 6 x 10⁴ cells/well where 10 µg/ml of 1a3 (closed square) and mouse IgG2a (open square) are immobilized, at 37 °C in a CO_2 incubater. Culture supernatants were collected at the indicated time points.

mAb should be specific, because liquid-phase 1a3 (simple co-culture) did abrogate HLA-DQ-restricted T-cell clonal responses (DT13.2), but not HLA-DR-restricted responses (BC20.7). However, solid-phase 1a3 markedly stimulated monocytes to produce IL-1β, IL-6, IL-10, IL-12 (p40 + p70), TNF-α and GM-CSF, whereas Ig subclass-matched control (mouse IgG2a) did not, as shown in Fig. 1B.

Activation of MAP kinases by anti-HLA mAbs

We next examined the effects of various inhibitors for signal transduction molecules. As shown in Fig. 2A, PD98059 (MEK-1 inhibitor) and SB203580 (p38 inhibitor) inhibited anti-DR-induced IL-1β production from monocytes. Genistein exhibited bi-phasic effect and inhibited IL-1β production at high concentrations (500 μM). We then studied the phosphorylation of various kinases by cross-linking class II HLA, among which only MAP kinases exhibited differential activation by anti-DR, -DQ and -DP. We stimulated monocytes directly with solid-phase anti-HLA mAbs, and cell lysates were subjected to Western blot analysis, using Abs to phosphorylated forms of Erk, JNK and p38 (anti-pErk, anti-pJNK and anti-pp38, respectively). As shown in Fig. 2B and 2C, Erk, especially Erk2, was phosphorylated only by anti-DR mAb (very weak phosphorylation was detected by anti-DQ or anti-DP, in the original film), whereas p38 was phosphorylated by anti-DR, anti-DQ and anti-DP mAbs.

Induction of monokine secretion from peptide-pulsed monocytes, using emetine-treated T cells of various HLA-restriction patterns

We wanted to determine if natural TCR-peptide-HLA interactions would induce monokine secretion by signaling through class II HLA molecules. T cell clones of various HLA-restriction patterns were treated with *de novo* protein synthesis inhibitor emetine. This is because it is highly likely that T-cell membrane proteins or T-cell soluble factors newly synthesized after activation, work in turn on monocytes. As shown in Table 1, three

human Th0 clones of distinct HLA restriction patterns, BC20.7 (BCGa-specific, DR14-restricted), DT13.2 (*Der f* I-specific. DQ6-restricted) and OT1.1 (p53-specific, DP5-restricted), were used for emetine-treatment, seven days after the last antigenic stimulation. First, we determined the ED50 of each clone to be 0.008 μM, 0.18 μM and 0.10 μM, for BC20.7, DT13.2 and OT1.1, respectively (not shown). Monocytes were pulsed with peptides, the concentrations of which were 625-fold as much as the ED50 (5 μM, 112.5 μM and 62.5 μM, for BC20.7, DT13.2 and OT1.1, respectively), followed by co-culture with emetine-treated T cells. These peptide concentrations induced plateau responses of monokine. Peptide-pulsed monocytes co-cultured with emetine-treated T cells, as shown in Table 1, produced IL-1β, IL-6, IL-10, IL-12 (p40 + p70), GM-CSF and TNF-α. It is noteworthy that the DR14-restricted clone, BC20.7, tends to induce pro-inflammatory monokines, such as IL-1β (105 pg/ml) and TNF-α (887 pg/ml) with the IL-10 / IL-1β ratio being 1.6, whereas DQ6-restricted clone, DT13.2, and DP5-restricted clone, OT1.1, tend to induce anti-inflammatory monokine IL-10 (787 pg/ml and 725 pg/ml, respectively) with the IL-10 / IL-1β ratio being 32.8 and 34.5 for DT13.2 and OT1.1, respectively. Allogeneic monocytes that do not share restriction HLA molecules, exhibited marginal monokine production in the presence of emetine-treated T cells.

Effects of protein kinase inhibitors on monokine productions

We co-cultured peptide-pulsed monocytes and emetine-treated BC20.7 T cells in the presence of several kinase inhibitors. These inhibitors were dissolved in DMSO and added to the culture medium at a final content of 0.5%, a content which did not inhibit DR-mediated monokine production. All data (not shown) collectively suggest that: (*a*) p38 is involved in both IL-1β and IL-10 production induced by ligating DR molecules expressed on monocytes; (*b*) MEK-1-Erk pathway is only partially involved in IL-1β production, being independent from p38-associated IL-1β

A

B

C
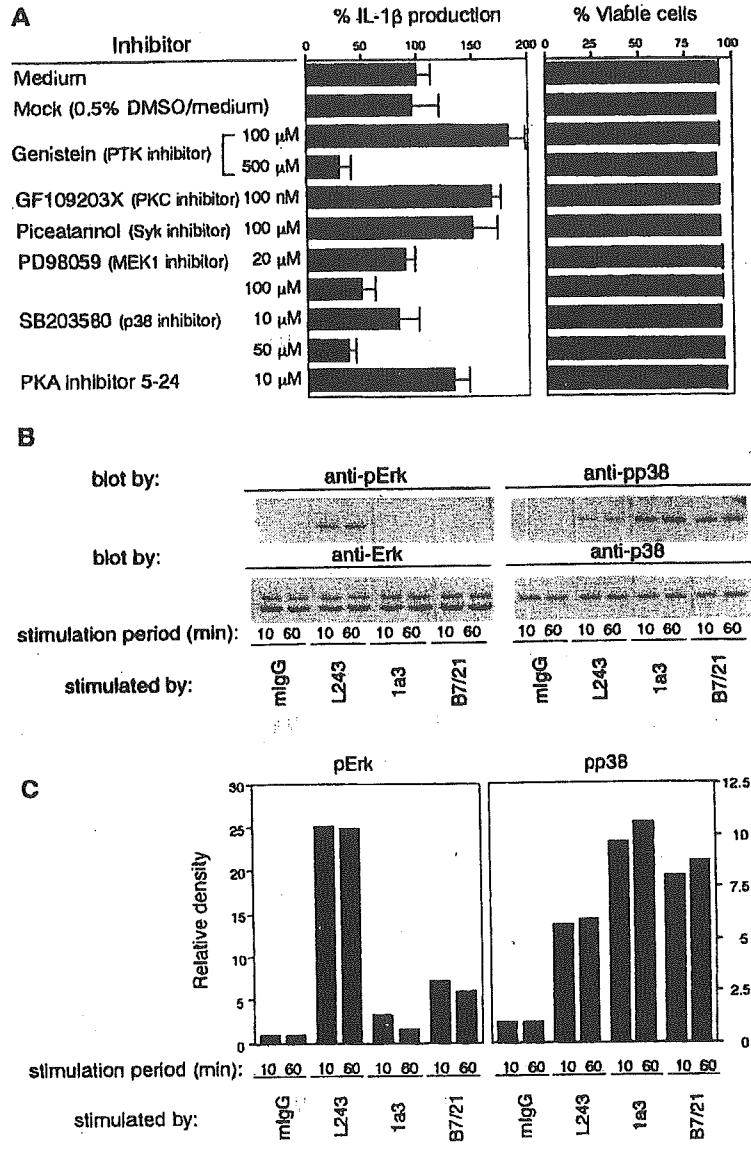
Figure 2. Activation of MAP kinase by mAbs to class II HLA. (A) Monocytes were co-cultured for 16 h on anti-class II coated plates, with the indicated inhibitors, at the indicated concentrations. Culture supernatants were collected and stored in aliquots at -80°C until determinations of cytokine concentrations. One hundred % IL-1β production was 205 pg/ml. Viable cell contents were determined using trypan blue. (B) After 10 and 60 min of stimulation with solid-phase mAbs, monocytes were lysed in 50 μl of lysing buffer, which were subjected to Western blot analysis either with Abs specific for Erk and p38, or with activated form of Erk and p38. (C) Relative densities are shown based on B.

Table 1.   Monokine production from monocytes stimulated with emetine-treated T cells + peptide.

| | T cell clones for stimulation | | |
Monokine	BC20.7	DT13.2	OT1.1
IL-1β	105.0 (pg/ml)	24.0	21.0
IL-6	87.5	62.5	140.0
IL-10	172.5	787.5	725.0
IL-18	<15.0	<15.0	<15.0
IL-12 (p40 + p70)	145.0	75.0	20.5
GM-CSF	475.0	1150.0	887.5
TNF-α	887.5	642.5	230.0
IL-10 / IL-1β	1.6	32.8	34.5

Emetine-treated T cells (BC20.7, DT13.2 and OT1.1) were cultured with peptide-prepulsed monocytes. The concentration of the peptides for each clonal responses was 625-fold as much as the ED50 (5 μM, 112.5 μM and 62.5 μM for BC20.7, DT13.2 and OT1.1, respectively). Culture supernatants after 16- (for IL-12), 24- (for IL-1β, IL-10, IL-18, GM-CSF and TNF-α) and 48-h (for IL-6) incubation were collected, and subjected to ELISA. Results are expressed as the mean value of triplicate determinations. Standard error was less than 20%.

production; and (c) activation of Erk may inhibit p38-mediated IL-10 production (Fig. 3).

Restriction molecules and cytokine-production patterns of short-term T cell lines

If the phenomenon observed earlier in this study occurs in a local milieu of T cell differentiation, lymphokine production patterns of T cells would be affected by restriction HLA molecules. Then, we next examined the production of IFN-γ and IL-4 from the *Der f*-specific T cell lines. As shown in Figure 4A, DR-restricted T cell lines produced more IFN-γ than IL-4, but DP-restricted T cell lines did more IL-4 than IFN-γ (p = 0.02 and 0.04 in

donor MA and NI, respectively). MAbs used in this study did not induce monokine secretion, when used as a soluble form (not shown). HLA types of MA (HLA-DRB1*1502-DRB5*0102-DQA1*0103-DQB1*0601 / HLA-DRB1*1405-DRB3*0202-DQA1*0101DQB1*0503) and NI (HLA-DRB1*0901-DRB4*0101-DQA1*0301-(HLA-DRB1*0901-DRB4*0101-DQA1*0301-DQB1*0303 / HLA-DRB1*1302-DRB3*0301-DQA1*0102-DQB1*0605) were distinct. Then, we examined the production of IFN-γ and IL-4 from the PPD-specific T cell lines (Fig. 4B). DR-restricted T cell lines produced more IFN-γ than IL-4, but DQ-resricted lines did more IL-4 than IFN-γ (p = 0.04 in donor MA). We next used X19 (random 19-mer peptide) to confirm
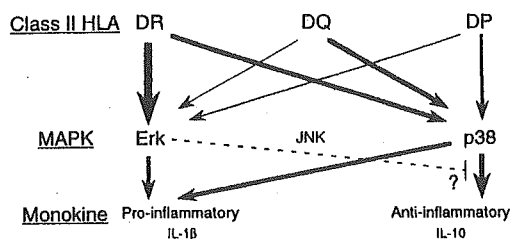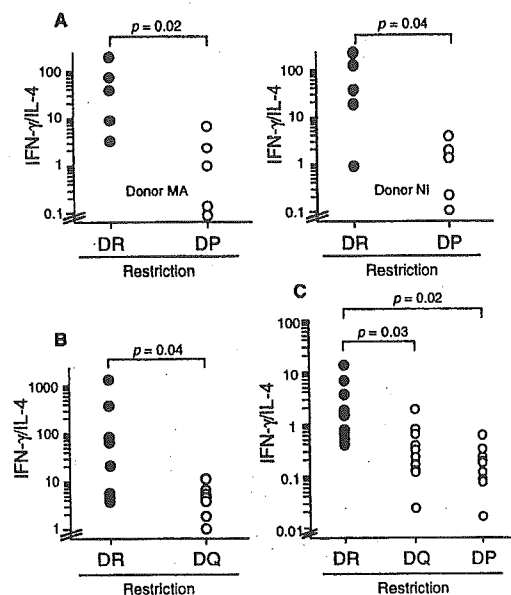
Figure 3. Summary of class II HLA-mediated MAPK activation. Note that other class II signaling elements that can be additive or modify the signaling via MAP kinases are not illustrated.


Figure 4. Restriction molecules and cytokine-production patterns of short-term T cell lines. *Der f* (crude mite antigen)-specific short-term T cell lines (A), PPD-specific short-term T-cell lines (B) and X19-reactive T cell clones (C) of various restriction patterns were restimulated with excess concentrations of antigens (A and B: 10 µg/ml, C: 500 µM), then after 48-h incubation, culture supernatants were collected for measurements of IFN-γ and IL-4 production by ELISA. One spot indicates one cell line.

the phenomenon observed in earlier studies, because: (a) DQ-restricted / *Der f*-reactive and DP-restricted / PPD-reactive T cells were not readily established; and (b) X19 can stimulate most CD4+ memory T cells to proliferate in the presence of cytokines, under cloned conditions. Indeed, DR-, DQ-, and DP-restricted T cell lines were obtained, the cytokine profiles of which again exhibited the similar results (Fig. 4C). Moreover, we titrated down the peptide concentration for DR-restricted responses and found that lower concentrations of X19 peptide did not lead to DQ/DP-restricted patterns of cytokines (not shown), which was indeed the case when emetine-treated BC20.7 T cells were incubated with monocytes in the presence of lower concentration of the antigenic peptide (not shown). These data indicate that DR-restricted and already activated peripheral CD4+ T cells carry Th1-prone phenotype, compared with DQ- / DP-restricted T cells, although the segregation pattern is incomplete.

Cross-linking HLA-DR molecules on B cells induces increased production of IgM without inducing B-cell proliferation.

To test whether signals via class II HLA molecules would affect production of Igs, we first cross-linked class II HLA molecules on B cells by making use of anti-DR mAb-coated culture plates (21). The supernatant fluids of 5-day cultures were assayed for Ig concentrations, among which only IgM was markedly affected

by DR ligation. As shown in Fig. 5A, cross-linking DR molecules with anti-DR mAbs (L243 or HU4) on B cells induced IgM production, whereas isotype-matched mouse IgG did not do so, thereby indicating that signals transmitted by FcR are not involved. Similar results were obtained, using B cells from another subject carrying DRB1*1405/1502 (data not shown).

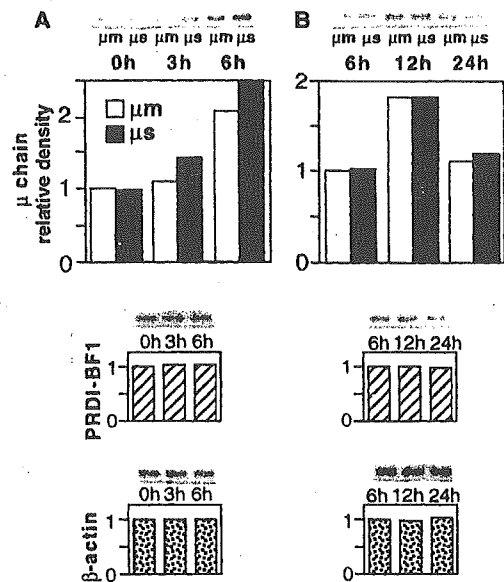**Cross-linking HLA-DR molecules enhances both membrane-type and secretory-type IgM heavy chain gene expression.**

To determine whether signals via DR molecules up-regulate µ chain mRNA, we cross-linked DR molecules on peripheral B cells (1 x 10^6) with either solid-phase anti-DR mAb
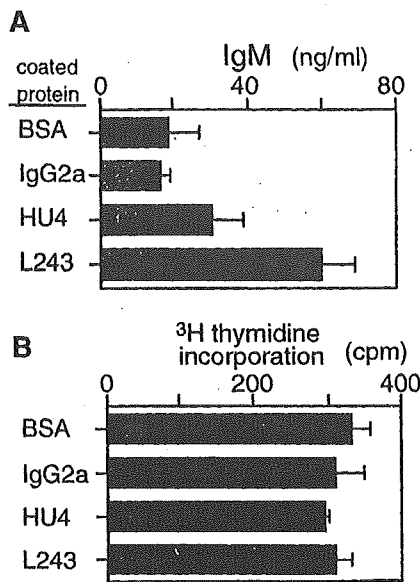
Figure 5. Cross-linking HLA-DR molecules on B cells induces increased production of IgM (*A*), without inducing proliferation (*B*). Mouse IgG2a, anti-DR mAb HU4, anti-DR mAb L243, or BSA were coated onto 96-well flat-bottomed culture plates at 10 μg / ml PBS. Purified peripheral B cells were incubated at 5×10^4 cells/well where mAbs are coated, at 37 °C in a CO_2 incubator for 3 days (proliferation assay) or for 5 days (IgM determination). HLA type of the B-cell donor was DRB1*0101/1201. Mean cpm of triplicate responses ± SD is indicated.

Figure 6. Cross-linking DR molecules enhances μ chain mRNA expression. Purified peripheral B cells were incubated in 24-well flat-bottomed culture plates at 1×10^6 cells/well where mAbs are coated, at 37 °C in a CO_2 incubator for 0, 3 and 6 h (*A*) or 6, 12 and 24h (*B*). As described under *experimental procedures*, RT-PCR and Southern blot analysis were done for membrane-type μ chains (μm; open columns), secretory-type μ chains (μs; closed columns), PRDI-BFI (hatched columns) and β-actins (shaded columns). mRNA expression levels were quantified using NIH image and represented by relative values compared with those from 0-h membrane-type μ chain, 0-h PRDI-BF1, or 0-h β–actin (A) and 6-h membrane-type μ chain, 6-h PRDI-BF1, or 6-h β–actin (B). HLA type of the B-cell donor was DRB1*0101/1201.

(L243) or solid-phase mouse IgG. Due to limitations in the number of purified B cells, we could test only 3 samples at one time. At 0, 3 and 6 h (Fig. 6A), or 6, 12 and 24 h (Fig. 6B) after the initiation of culture, B cells were analyzed for mRNA expression for μ chains, using RT-PCR and Southern blot analysis. Relative mRNA level was analyzed. When we tested the kinetics, μ chain mRNA increased in a time-dependent fashion (Fig. 6A), and reached maximum at 12 h (Fig. 6B). This increase was not due to the enhanced recovery of mRNA, as evidenced by the presence of an equal amount of β-actin mRNA in each sample. The μ chain mRNA level induced by control mouse IgG at 3, 6, 12 and 24 h was practically the same as that induced by anti-DR mAbs at 0 h (data not shown).

To test whether the DR-generated signal

induced differentiation of B cells to plasma cells, we analyzed PRDI-BF1 transcripts. PRDI-BF1 is a human homologue of Blimp-1, the expression of which is characteristic of late B cells and plasma cells (22,23). However, as shown in Fig. 6, DR-generated signals up-regulated no mRNA for PRDI-BF1. The presence of PRDI-BF1 transcripts is indicative of the presence of plasma cells in this cell preparation. These data suggest that IgM production induced by cross-linking of DR molecules is regulated at the mRNA level, and is not associated with B-cell differentiation to plasma cells.

Emetine-treated and HLA-DR-restricted T cells are capable of inducing IgM production by B cells

Although earlier observations strongly suggest that the ligation of HLA-DR molecules directly stimulates B cells to produce IgM, the outcome of ligation by mAbs should be affected by epitopes recognized by these mAbs and their affinity. Indeed, anti-HLA-DR mAb HU-4, exerted weaker effects than did L243 (Fig. 5). It is unlikely that HLA-DRB4 molecules recognized by L243 are transmitting the signals, because the B-cell donor in Figures 5 and 6 did not carry DRB4-positive haplotypes. Therefore, we next asked if a similar phenomenon occurs, on natural TCR-peptide-HLA interactions. An HLA-DR-restricted T cell clone was treated with the de novo protein synthesis inhibitor emetine, because it is highly likely that T-cell membrane proteins or T-cell soluble factors newly synthesized after activation by peptide-pulsed B cells, work on B cells. Under conditions where T cells are treated with 90 μg / ml emetine for 1 h followed by co-culture with peptide-pulsed B cells bearing restriction HLA molecules, T cells produced <25 pg / ml of IL-4, whereas non-treated T cells produced 3580 pg / ml of IL-4, although cell-surface TCR remains practically

the same level (data not shown), indicating that de novo protein synthesis of T cells is abrogated by emetine. A T-cell clone BC20.7 (BCGa-specific, DR14-restricted) and B cells purified from PBMC of the donor of BC20.7, was used in subsequent experiments. As shown in Table 2, levels of IgM, IgG1, IgG4, IgE, and IgA were detected when mock-pulsed B cells were co-cultured with emetine-treated T cells. However, when B cells were pre-pulsed with the antigenic peptide, marked enhancement of IgM and marginal enhancement of IgA production were observed and such was not the case when peptide-pulsed B cells were cultured in the absence of T cells (not shown).

## Cross-linking DR molecules on B cells up-regulates Syk kinase activity

To investigate possible protein tyrosine phosphorylation associated with this event, detergent lysates of peripheral B cells and LD2B cells treated with anti-DR mAb or control mouse IgG, were analyzed. Fig. 7 shows that protein-tyrosine phosphorylation was enhanced by cross-linking of DR molecules on peripheral B cells (Fig.7A, lane 2 vs 1, 3). Bands corresponding to proteins with an approximate molecular mass of 65, 70, 110 and

Table 2. Ig production from B cells induced by a DR-restricted T-cell clone.

peptide	IgM	IgG1	IgG2	IgG3	IgG4	IgE	IgA
							ng / ml
-	37.5	124.5	<3.1	<3.1	3.13	31.2	480
+	223.5	96.0	<3.1	<3.1	1.41	21.2	980

B cells either mock-pulsed or pulsed with BCGap84-100 were cultured with an HLA-DR14 (DRB1*1405)-restricted and emetine-treated T cell clone BC20.7 for 5 days. B cells were purified by, and the T-cell clone was established from a donor carrying DRB1*1405/1502. Mean values of duplicate determinations are indicated. SD was less than 25%.
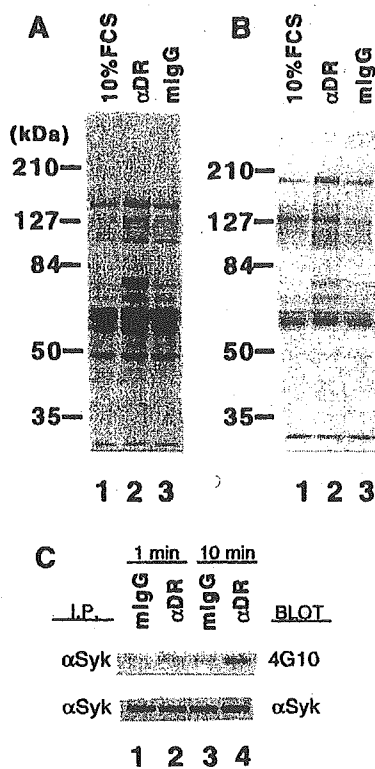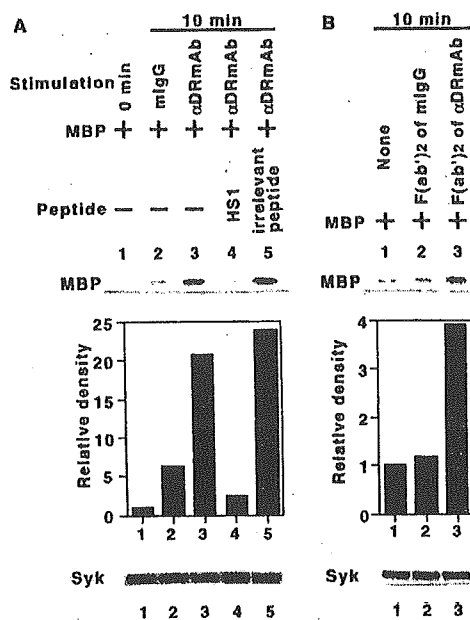
Figure 7. Cross-linking DR up-regulates protein-tyrosine phosphorylation of Syk. The peripheral B cells (*A*) and LD2B B cells (*B* and *C*) were incubated with biotinylated anti-DR mAb, L243 or biotinylated mouse IgG followed by avidin for 10 min (*A* and *B*) or for 1 and 10 min (*C*), then cells were lysed. Cell lysates were either immunoprecipitated by anti-Syk Ab (*C*) or directly (*A* and *B*) resolved by 9 % SDS-PAGE, and then transferred to nitrocellulose membranes. Protein-tyrosine phosphorylation was detected by using anti-phosphotyrosine mAb (4G10) and ECL.

130 kDa were reproducibly hyper-phosphorylated. Likewise, cross-linking of DR molecules on LD2B induced tyrosine-phosphorylation of 65 and 70 kDa proteins (Fig. 7B, lane 2 vs 1, 3). Furthermore, immunoprecipitation by anti-Syk Ab followed by blotting with anti-phosphotyrosine mAb 4G10, exhibited anti-DR-induced tyrosine phosphorylation of Syk molecules expressed in

LD2B cells (Fig. 7C).

To further confirm that Syk is activated directly via HLA-DR, Syk kinase activity was determined by in vitro kinase assay, using Syk molecules immunoprecipitated with anti-Syk Ab, and MBP as a substrate. Because a large number of B cells are required for immunoprecipitation followed by in vitro Syk kinase assay, we used a B lymphoblastoid cell line LD2B homozygous for DRB1*1501, which secretes IgM in the absence of specific stimuli. LD2B was selected among many B cell lines because (a) anti-DR-induced phosphorylation pattern of LD2B was similar to that of peripheral B cells, including the phosphorylation of 70-kDa protein (Fig. 7); and (b) LD2B B cells expressed IgM heavy chain genes. Enhancement of IgM production from LD2B B cells after cross-linking DR molecules was only marginal, probably because LD2B cells constitutively showed a 50- to 80-fold higher IgM secretion than did peripheral B cells, on a single cell basis (data not shown). As shown in Fig. 8A, however, cross-linking of DR molecules by biotinylated anti-DR mAb plus avidin, induced marked phosphorylation of MBP (*lane* 3), whereas incubation of LD2B with biotinylated mouse IgG + avidin, only marginally induced phosphorylation of MBP (*lane* 2 vs 1). Because MBP is not a substrate specific for Syk kinase, and it might be that MBP was phosphorylated by certain kinases co-precipitating with Syk, we also asked if the effect of Syk on MBP would be inhibited competitively by HS1p388-402 peptide, a substrate specific for Syk. In vitro Syk kinase assay with MBP (2 µg/sample; 0.11 nmol/sample) was done in the presence of either a 250-fold molar excess of the HS1 peptide (27.5 nmol/sample) or an irrelevant peptide carrying two tyrosine residues (EIKYNGEEYLIL; 27.5 nmol/sample). Indeed, MBP phosphorylation was inhibited by the HS1 peptide, but not by the irrelevant peptide (*lanes* 4 and 5).

It is also important to note that Syk molecules are associated with FcγR and are activated by cross-linking of the receptor (24,25). It is therefore conceivable that the increment in Syk kinase activity we observed

Figure 8. Cross-linking DR up-regulates Syk kinase activity. LD2B B cells were incubated with biotinylated anti-DR mAb L243 or biotinylated mouse IgG followed by avidin for 10 min, then cells were lysed. Lysates were immunoprecipitated with anti-Syk Ab. (A) An aliquot of immunoprecipitated proteins were immunoblotted with anti-Syk Ab. Residual Syk proteins on agarose beads were used for in vitro immune complex kinase assay, using MBP as a substrate. The HS1 peptide or an irrelevant peptide was added to the assay. (B) LD2B B cells were incubated with biotinylated F(ab')2 of anti-DR mAb L243, or biotinylated F(ab')2 of control mouse IgG followed by avidin for 10 min. Cells were lysed, immunoprecipitated, and subjected to in vitro Syk kinase assay. MBP phosphorylation levels were quantified using a bio-imaging analyzer (BAS2000, Fuji Film, Tokyo), and represented by relative values compared with those of 0 min (A) or 10 min (B) (unstimulated cells).

may be due to cross-reaction of mouse Ig with human FcγR expressed on B cells. To exclude this possibility, we prepared a biotinylated F(ab')2 fragment of anti-DR mAb L243 or that of control mouse Ig. As shown in Fig. 8B, cross-linking of F(ab')2 fragment of anti-DR mAb L243 induced phosphorylation of MBP (lane 3), whereas F(ab')2 fragment of mouse Ig induced little phosphorylation of MBP (lane 2), compared with a control (avidin only; lane 1).

This indicates that Syk phosphorylation is induced by cross-linking DR but not FcγR. These differences in phosphorylation patterns were not due to the enhanced recovery of these kinases, as evidenced by the presence of an equal amount of Syk protein molecule in each sample (Fig. 8A and 8B). These data are consistent with results obtained using the Syk inhibitor piceatannol on IgM production, thereby collectively indicating that HLA-DR molecules on B cells not only present antigenic peptides to T cells, but also up-regulate IgM production, in association with Syk activation and without the involvement of Src kinases.

Kinetics of proliferation induced by cross-linking HLA-DR molecules on CD4+ T cells

We investigated kinetics of T-cell proliferation when HLA-DR molecules on CD4+ T cell were cross-linked by solid-phase mAbs (Fig. 9). Cross-linking by anti-HLA-DR mAb L243 stimulated T cells to proliferate while control Ab did not do so. The proliferative response reached maximum at 24 hr, as did the response induced by anti-CD3 mAb. T cells incubated with biotinylated anti-DR + avidin proliferated as well (data not shown). However, T cells stimulated by biotinylated anti-DR alone did not do so (data not shown). Thus not only binding of anti-DR mAb molecules but also their cross-linking is necessary for T cells to proliferate (25).

T cell clonal anergy induced by a soluble form of antigenic peptide, anti-CD3 mAb and anti-HLA-DR mAb

SF36.16 cells cultured with the soluble form of antigenic peptide for 7 days resulted in low responsiveness to irradiated PBMC in the presence of the indicated concentration of peptide (Fig. 10A). Thus, when these cells are re-stimulated with 1 nM antigenic peptide and irradiated autologous PBMC, there maybe only marginal proliferation, whereas SF36.16 cells cultured for 7 days with irradiated autologous PBMC prepulsed with 5 μM peptide did exhibit marked proliferative responses when re-stimulated with 1 nM peptide and irradiated
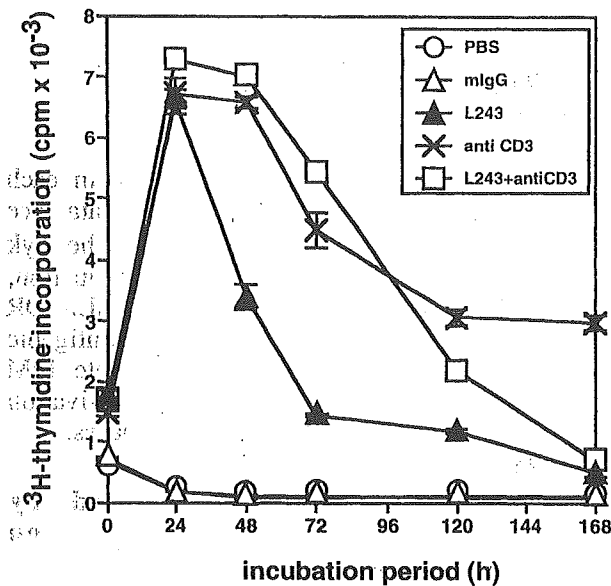
Figure 9. Kinetics of proliferation induced by cross-linking HLA-DR molecules on CD4⁺ T cells. Kinetics of T-cell proliferation was investigated when HLA-DR or CD3 molecules on CD4⁺ T cell were cross-linked by solid-phase mAbs (open circle, PBS; open triangle, control mIgG; closed triangle, anti-HLA-DR mAb L243; cross, anti-CD3 mAb OKT3; open square, L243 + OKT3). After indicated incubation periods (0-168 hr), T cells were cultured in the presence of 1 μCi/well [³H]thymidine during the final 16-h period, and the incorporated radioactivity was measured by scintillation counting. All data are expressed as the mean value of duplicate determinations ± standard error. The experiment shown is representative of two independent experiments.

autologous PBMC (Fig. 10A).

SF36.16 cells cultured in the presence of solid-phase anti-CD3 mAb OKT3 for 7 days also showed low responsiveness (Fig. 10B). Interestingly, SF36.16 cells cultured in the presence of solid-phase anti-HLA-DR mAb L243 for 7 days also showed a low response (Fig. 10C). Furthermore, when exogenous IL-2 at 200 U/ml was added to re-stimulation the culture, the responsiveness was restored ($p = 0.0041$), although human recombinant IL-2 only marginally exhibited an enhancing effect in control cultures ($p = 0.25$; Fig. 10D). All these observations indicate that L243-stimulated T cells were rendered anergic. It is unlikely that

solid-phase L243 blocked antigen presentation to T cells in the re-stimulation culture, because levels of responsiveness were the same, between L243-stimulated and control cultures, in the presence of IL-2 ($p = 0.11$; Fig. 10D).

Expression of CDK inhibitors p21^{Cip1} and p27^{Kip1} in anergic CD4⁺ T cells

As shown in Fig. 11, T cells with clonal anergy induced by stimulation with the soluble form of an antigenic peptide, expressed higher level of CDK inhibitor p27^{Kip1} on day7, than did those cultured with irradiated autologous PBMC prepulsed with 5 μM agonistic peptide. Likewise, anergic T cells induced by cross-linking its CD3 or HLA-DR, expressed higher level of CDK inhibitor p27^{Kip1} . In contrast, both expressed the same levels of CDK inhibitor p21^{Cip1} (Fig. 11), p15^{INK4b} and p16^{INK4a} (data not shown). Neither T cells stimulated with control IgG (Fig. 9) nor those stimulated with peptide-unpulsed PBMC showed proliferative responses. Therefore, T cells under such stimuli died on day 7, which did not enable us to test protein expression.

Cytokine production from fibroblasts by stimulus via HLA-II molecules

Since we did not observe proliferative responses of T cells when fibroblasts were used as APC, we then considered the possibility that HLA-DR molecules on fibroblasts may act as receptor molecules (27). The cytokine production from PDL was first evaluated by determining cytokine levels in culture supernatants, when fibroblasts were stimulated via HLA-DR molecules by anti-HLA-DR mAbs. The isotype-matched immunogloblin was used as a control. As shown in Figure 12, stimulated PDL produced larger amounts of IL-8, IL-6, MCP-1 and RANTES compared with control cells.

We then examined whether cytokine production from fibroblasts actually occurred when HLA-DR molecules on fibroblasts are ligated by their natural ligands. To address this issue, peptide pulsed fibroblasts were co-cultured with emetine-treated T cells. Since
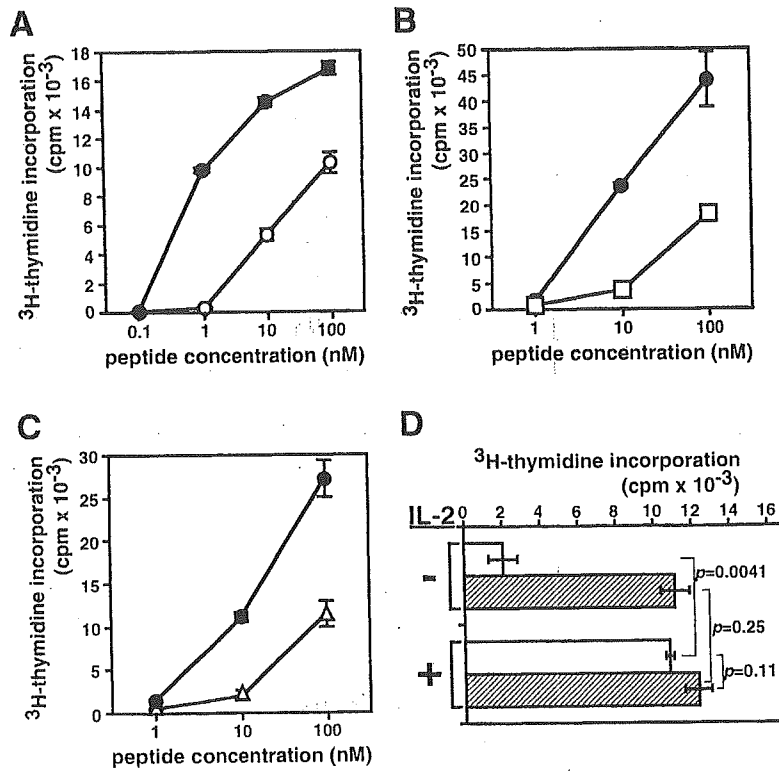
Figure 10. T cell clonal anergy induced by a soluble form of the antigenic peptide (A), anti-CD3 mAb (B) and anti-HLA-DR mAb (C, D). SF36.16 T cells were primarily stimulated with irradiated autologous PBMC prepulsed with the wild-type peptide (5 μM BCGap84-100 for 5 h at 37 °C; closed circle or hatched bar), soluble wild-type peptide (1 μM BCGap84-100; open circle), plastic immobilized anti-CD3 (OKT3; open square) or plastic immobilized anti-DR (L243; open triangle or open bar). Seven days after initiation of culture, T cells in these cultures were re-stimulated (secondary stimulation) with various concentrations of wild-type BCGap84-100 plus irradiated PBMC (A, B, C), or with 10 nM wild-type BCGap84-100 plus irradiated PBMC, in the presence or absence of 200 U/ml of exogeneous human recombinant IL-2 (D). The T cells were cultured on a 96-well plate for 72 h, and subjected to [^{3}H]thymidine incorporation assay. All data are expressed as the mean value of duplicate determinations ± standard error. Data are representative of two independent experiments. (D) Proliferative response of T cells to exogeneous IL-2. Primary stimulation, secondary stimulation and [^{3}H]thymidine incorporation assay were done as described above. Results are expressed as the means ± SD of duplicate measurement. Statistical significance was analyzed using Student's t test.

emetine is a *de novo* protein synthesis inhibitor, we first treated T cells with emetine to prevent T cells from up-regulating cytokines or cell-surface molecules. As shown in Figure 13, IL-8, IL-6, MCP-1 and RANTES were indeed produced from peptide-pulsed PDL following

16 hr culture (1×10^4 cells/well) with emetine-treated T cells, whereas no cytokines were produced when the cells were merely pulsed with peptide. Peptide-pulsed and IFN-γ treated PDL produced larger amounts of cytokines including IL-8, IL-6, MCP-1 and RANTES