The Journal of Immunology

Monocytes Are Differentially Activated Through HLA-DR, -DQ, and -DP Molecules Via Mitogen-Activated Protein Kinases

Takako Matsuoka, Hiroki Tabata and Sho Matsushita

J Immunol 2001; 166:2202-2208; ;
doi: 10.4049/jimmunol.166.4.2202
http://www.jimmunol.org/content/166/4/2202

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Monocytes Are Differentially Activated Through HLA-DR, -DQ, and -DP Molecules Via Mitogen-Activated Protein Kinases[1]

Takako Matsuoka, Hiroki Tabata, and Sho Matsushita[2]

When HLA-DR, -DQ, and -DP were cross-linked by solid-phase mAbs, monocytes produced monokines and only anti-DR markedly activated mitogen-activated protein (MAP) kinase extracellular signal-related kinase, whereas anti-DR, anti-DQ, and anti-DP all activated MAP kinase p38. Activation of extracellular signal-related kinase was not inhibited by neutralizing Ab to TNF-α. Anti-DR and DR-restricted T cells stimulated monocytes to produce relatively higher levels of proinflammatory monokines, such as IL-1β, whereas anti-DQ/DP and DQ-/DP-restricted T cells stimulated higher levels of anti-inflammatory monokine IL-10. IL-10 production was abrogated by the p38 inhibitor SB203580, but rather enhanced by the MAP/extracellular signal-related kinase kinase-I-specific inhibitor PD98059, whereas IL-1β was only partially abrogated by SB203580 and PD98059. Furthermore, DR-restricted T cells established from PBMC, which are reactive with mite Ags, purified protein derivative, and random 19-mer peptides, exhibited a 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. ***The Journal of Immunology,*** **2001, 166: 2202–2208.**

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: 1) cross-linking class II molecules induces an increase in intracellular calcium and cAMP in mouse or human B cell lines (2–5); 2) class II MHC-mediated signals lead to homotypic aggregation of B cells (6); 3) cross-linking HLA-DR molecules on B cells induces apoptosis (7); 4) 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); and 5) cytoplasmic domain mutants of class II MHC abrogate generation of intracellular cAMP (9) and translocation of protein kinase C (PKC)[3] (10). Furthermore, by using human peripheral blood monocytes, cross-linking HLA-DR molecules with staphylococcal enterotoxins induces phosphorylation of Src family kinases (Lyn, Fgr) (11). Another study by Kanner et al. (12) demonstrated that ligation of class II activates Syk and ZAP-70 in B cells and activated human $CD4^+$ T cells, respectively . Moreover, engagement of class II molecules on the THP-1 monocyte cell line with staphylococcal enterotoxin A induced IL-1β and TNF-α (13). Although 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 functions as an Ir gene for schistosomal Ag-specific immune responses, whereas HLA-DQ functions as an Is gene, being epistatic to DR. However, their roles in activating Th1/Th2/APC have remained elusive. To investigate the consequence of signaling events through distinct subregion products of class II HLA, we tested monokine secretion patterns induced by 1) solid-phase mAbs to HLA-DR, -DQ, and -DP molecules expressed on peripheral blood-adherent monocytes and 2) coculture of peptide-pulsed monocytes with emetine-treated T cell clones of various HLA-restriction patterns.

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),

Department of Neuroscience and Immunology, Division of Immunogenetics, Kumamoto University Graduate School of Medical Sciences, Honjo, Kumamoto, Japan

Received for publication July 3, 2000. Accepted for publication November 15, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by grants-in-aid from the Ministry of Education, Science, Sports and Culture, Japan, a Research Grant for Intractable Diseases from the Ministry of Health and Welfare, Japan, the Sagawa Cancer Research Foundation, Osaka Cancer Research Foundation, and the Mochida Memorial Foundation. T.M. is a Research Fellow supported by the Japan Society for the Promotion of Science.

[2] Address correspondence and reprint requests to Dr. Sho Matsushita, Department of Neuroscience and Immunology, Division of Immunogenetics, Kumamoto University Graduate School of Medical Sciences, 2-2-1 Honjo, Kumamoto 860-0811, Japan. E-mail address: imgshom@gpo.kumamoto-u.ac.jp

[3] Abbreviations used in this paper: PKC, protein kinase C; PPD, purified protein derivative; *Der f, Dermatophagoides farinae*; Erk, extracellular signal-related kinase; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; MEK, MAP/Erk kinase; BCG, bacillus Calmette-Guérin; PTK, protein tyrosine kinase.

0022-1767/01/$02.00

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, Manchester, U.K.) were purchased. Mouse IgG, IgG1, and IgG2a were purchased for control from BioPur AG (Bubendorf, Switzerland) and Biogenesis (Poole, U.K.). Igs were purified from the ascites form of mAbs using a protein A column (Pierce, Rockford, IL). $F(ab')_2$ 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, MO), GF109203X (Sigma), piceatannol (Sigma), PD98059 (New England Biolabs, Beverly, MA), and SB203580 (Calbiochem, La Jolla, CA) were purchased. *Dermatophagoides farinae* (*Der f*) Ags were kindly provided by Torii Pharmaceuticals (Tokyo, Japan). Purified protein derivative (PPD) was purchased from the Japan BCG Laboratory (Tokyo, Japan). Peptides with defined sequences were synthesized using a solid-phase simultaneous multiple peptide synthesizer PSSM-8 (Shimadzu, Kyoto, Japan) based on the F-moc strategy and using a 10-fold molar excess of single F-moc amino acids, then were purified using C18 reversed-phase HPLC. In the case of degenerate peptides, the introduction of randomized sequence positions was done in a double coupling step with equimolar mixtures of F-moc-L-amino acids used in an equimolar ratio with respect to coupling sites of the resins (all positions have 19 amino acid residues, except for Cys).

Human T cell clones

Human $CD4^+$ T cell clone BC20.7 that recognizes DR14 (DRA + DRB1*1405) + residues 84–100 of bacillus Calmette-Guérin (BCG) a protein (BCGap84–100; EEYLILSARDVLAVVSK) has been described previously (16). OT1.1 (17) and DT13.2 (1) are specific for DP5 (DPA1*0201 + DPB1*0501) + p53p153–165 (STPPPGTRVRAMAI YKQS) and DQ6 (DQA1*0102 + DQB1*0602) + *Der f* Ip18–31 (RSL-RTVTPIRMQGG), 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 (Life Technologies, Grand Island, NY) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% pooled, heat-inactivated normal human male plasma in 24-well flat-bottom 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, Piscataway, NJ). The PBMC were incubated at 3×10^7 cells in 10 ml of 10% human serum/RPMI 1640 for 1.5 h in 90-mm culture-grade plastic petri dishes precoated with heat-inactivated autologous plasma at 37°C in a CO_2 incubator. After removing nonadherent cells, the adherent cells were recovered from plates by incubating with ice-cold 0.05% EDTA/PBS for 10 min and repeated pipetting. Monocytes were cultured for 48 h to allow adherence-induced transcription of monokine mRNA to subside (18). This population was composed principally of monocytes and were >90% CD14 positive, as analyzed by FACS (data not shown). *HLA class II* (*DR*, *DQ*, and *DP*) alleles were determined as described elsewhere (1). HLA types of the two monocyte donors were DRB1*0101/1201 and DRB1*1405/1502, both of which are negative for DRB4.

Stimulation of monocytes

Ten micrograms per milliliter anti-DR Ab (L243), anti-DQ Ab (1a3), anti-DP Ab (B7/21), and mouse IgG (alternatively, IgG1 and IgG2a) were precoated onto 96-well flat-bottom culture plates. Adherent cells were incubated at 6×10^4 cells/well where mAbs are immobilized at 37°C in a CO_2 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) (19) for 1 h at 37°C were washed three times with RPMI 1640 medium. Cells were resuspended in culture medium, incubated for 3 h at 37°C, and then washed three times with RPMI 1640 medium and cocultured with peptide-pulsed or mock-pulsed monocytes. Culture supernatants after a 16-h (for IL-12), 24-h (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; 90 μg/ml emetine treatment resulted in a complete abrogation of IL-4 production, but not IL-12 produced by peptide-pulsed monocytes (data 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 for mock-pulsed monocytes stimulated with emetine-treated T cells (data 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 (data not shown).

ELISAs

The human IL-4, IFN-γ, IL-1β, 10, 12 (p40 + p70), GM-CSF, and TNF-α ELISA kits (BioSource International, Camarillo, CA) and human IL-6 ELISA kit (Genzyme, Cambridge, MA) were used for quantitation of lymphokines in the supernatants according to manufacturer's instructions. ELISA kit for IL-18 was kindly provided by M. Kurimoto (Hayashibara Biochemical Laboratories, 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 a 10-to 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 sodium orthovanadate, 1 mM NaF, and 5 mM EDTA plus a protease inhibitor mixture 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 and 0.2% Tween 20 in TBS, the membrane was incubated with Abs specific for extracellular signal-related kinase (Erk), c-Jun N-terminal kinase (JNK), and p38 (Santa Cruz Biotechnology, Santa Cruz, CA) or with Abs specific for the activated form of Erk, JNK, and p38 (Upstate Biotechnology, Lake Placid, 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 National Institutes of Health, Bethesda, MD, and available on 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 Ag)-specific short-term T cell lines were established from PBMC from two donors carrying different HLA types (donor MA, HLA-DRB1*1405/DRB1*1502; donor NI, HLA-DRB1*0901/DRB1*1302). HLA-DR-restricted and HLA-DP-restricted T cell lines were established by coculture 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 (data not shown), and all of the cell lines of expected restriction patterns were used for the analysis. These cell lines were restimulated with excess concentrations of Ags (10 μg/ml) and then after a 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 coculture 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 conditions.[4] Restriction molecules were determined by inhibition assays with mAbs.

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. 1*A*, the effect of the anti-DQ

[4] S. Matsushita, Y. Tanaka, T. Matsuoka, and T. Nakashima. Identification of peptide ligands recognized by single CD4T cells, using limiting dilution primary culture with randomized peptides followed by epitope scanning with combinatorial peptide libraries. *Submitted for publication*.

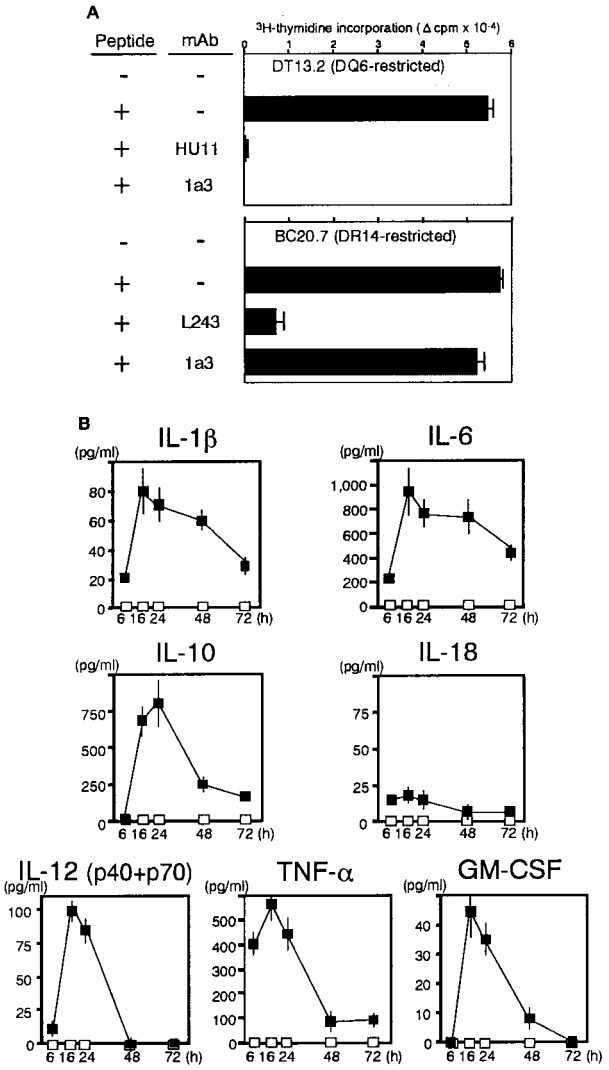

FIGURE 1. Monokine secretion induced by solid-phase mAbs to HLA. *A*, DT13.2 and BC20.7 were cultured in the presence of *Der f* 1 peptide (for DT13.2) or BCGa peptide (for BC20.7) and irradiated autologous PBMC, with or without anti-class II HLA mAbs. *B*, Adherent cells were incubated at 6×10^4 cells/well where 10 μg/ml of 1a3 (■) and mouse IgG2a (□) are immobilized at 37°C in a CO_2 incubator. Culture supernatants were collected at the indicated time points.

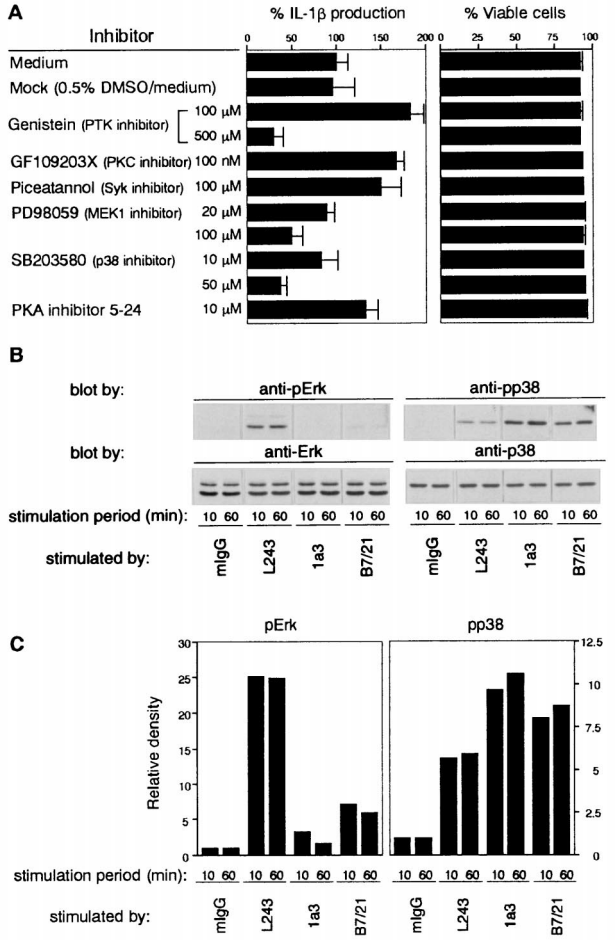

FIGURE 2. Activation of MAP kinases by mAbs to class II HLA. *A*, Monocytes were cocultured for 16 h on anti-DR (L243)-coated plates, with the indicated inhibitors, at the indicated concentrations. Culture supernatants were collected and stored in aliquots at −80°C until determinations of cytokine concentrations. One hundred percent 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 (anti-DR L243, anti-DQ 1a3, and anti-DP B7/21), monocytes were lysed and subjected to Western blot analysis either with Abs specific for Erk and p38 or with the activated form of Erk and p38. *C*, Relative densities are shown based on *B*.

mAb should be specific, because liquid-phase 1a3 (simple coculture) 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. 1*B*. IL-18 production was only marginal. The peaks of IL-1β, IL-10, IL-12 (p40 + p70), TNF-α, and GM-CSF secretion were at 16 or 24 h. On the other hand, the IL-6 concentration was sustained from 16 to 48 h. IL-12 (p40 + p70) showed its peak at 16 h and was no longer detectable at 48 h. We obtained similar results regarding specific inhibitory activity on Ag presentation and kinetics when mAbs to DR (L243) and DP (B7/21) were used (data not shown).

Activation of mitogen-activated protein (MAP) kinases by anti-HLA mAbs

We next examined the effects of various inhibitors for signal transduction molecules. As shown in Fig. 2*A*, PD98059 (MAP/Erk kinase (MEK)-1 inhibitor) and SB203580 (p38 inhibitor) inhibited anti-DR-induced IL-1β production from monocytes. Genistein exhibited a biphasic 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. 2, *B* and *C*, 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. Control mouse IgG did not induce phosphorylation of these kinases, and this was also the case when mouse IgG1 or IgG2a was used (data not shown). Phosphorylation of JNK molecules was barely detectable and was not enhanced by stimulation (data not shown). All of these observations indicate that signaling through DR molecules phosphorylates (i.e., activates) Erk and less efficiently p38, whereas signaling through DQ and DP activates p38 and much less efficiently Erk.

To confirm that MAP kinases activation is solely due to class II ligation, we examined the effects of $F(ab')_2$ of anti-DR mAb L243 and anti-TNF-α. As shown in Fig. 3*A*, $F(ab')_2$ of L243 markedly induced phosphorylation of Erk, whereas $F(ab')_2$ of control mouse IgG did not. Phosphorylation of Erk induced by $F(ab')_2$ of anti-DR mAb was not inhibited by 10 μg/ml neutralizing Ab to TNF-α (Fig. 3*B*). Moreover, as shown in Fig. 3*C*, the anti-TNF-α at 10 μg/ml inhibited the IL-1β production induced by 10 pg/ml TNF-α. Indeed, monocytes stimulated by $F(ab')_2$ of anti-DR mAb for 60 min never produced more than 5 pg/ml TNF-α (data not shown). Taken together, neither Fc of Ig nor TNF-α is likely to be involved in MAP kinase activation induced by class II ligation.

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

We wanted to determine whether 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 the 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 on monocytes. These T cell clones were not reactive to autoantigens or to alloantigens (1, 16, 17). As shown in Table I, 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 7 days after the last antigenic stimulation. First, we determined the ED_{50} of each clone to be 0.008, 0.18, and 0.10 μM for BC20.7, DT13.2, and OT1.1, respectively (data not shown). Monocytes were pulsed with peptides, the concentrations of which were 625-fold as much as the ED_{50} (5, 112.5, and 62.5 μM for BC20.7, DT13.2, and OT1.1, respectively), followed by coculture with emetine-treated T cells. These peptide concentrations induced plateau responses of monokine production and the kinetics of monokine production were practically the same as that observed with solid-phase mAbs shown in Fig. 1*B* (data not shown). Peptide-pulsed monocytes cocultured with emetine-treated T cells, as shown in Table I, 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 proinflammatory 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 the DQ6-restricted clone DT13.2 and DP5-restricted clone OT1.1 tend to induce anti-inflammatory monokine IL-10 (787 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. The magnitude of response was markedly different between Ab-induced (Fig. 1*B*) and T cell-induced (Table I) secretion of IL-6 and GM-CSF, the mechanisms for which are yet to be determined. Allogeneic monocytes that do not share restriction HLA molecules exhibited marginal monokine production in the presence of emetine-treated T cells, and neutralizing Abs to TNF-α (20), CD154 (21), and CD23 (22) only partially abrogated IL-12 or IL-10 production (data not shown).

Table I. *Monokine production from monocytes stimulated with emetine-treated T cells + peptide*[a]

Monokine	T Cell Clones (HLA Restriction) for Stimulation (pg/ml)		
	BC20.7 (DR14)	DT13.2 (DQ6)	OT1.1 (DP5)
IL-1β	105.0	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

[a] 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 response was 625-fold as much as the ED_{50} (5, 112.5, and 62.5 μM for BC20.7, DT13.2, and OT1.1, respectively). Culture supernatants after a 16-h (for IL-12), 24-h (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. SE was <20%.

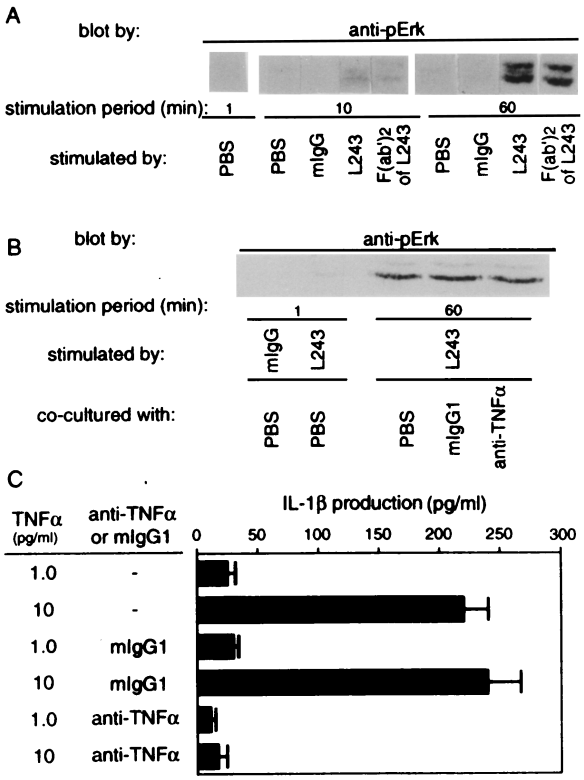

FIGURE 3. Fc and TNF-α are not involved in Erk and IL-1β activation induced by class II ligation. *A*, Ab preparations including $F(ab')_2$ of L243 were used for stimulation of monocytes, as for Fig. 2*B*. *B*, Anti-TNF-α at 10 μg/ml and controls were added to this culture system. *C*, TNF-α at indicated concentrations were cocultured with monocytes for 16 h in the presence of anti-TNF-α or control mIgG1 at 10 μg/ml, and IL-1β levels in culture supernatant fluids were determined.

When Western blot analysis was done using peptide-pulsed monocytes in the presence of emetine-treated T cells, signals of T cell origin could not readily be discriminated from those of monocytes (data not shown). When we examined the monokine secretion induced by cross-linking class II HLA molecules using solid-phase mAbs to HLA-DR, -DQ, and -DP, the IL-10:IL-1β ratio under DR, DQ, and DP stimulation was 1.8, 12.8, and 14.4, respectively, indicating that differential monokine production induced by stimulation through DR, DQ, and DP molecules is observed even when monocytes are directly stimulated by mAbs.

Effects of protein kinase inhibitors on monokine productions

We cocultured 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, as shown in Fig. 4*A*. The

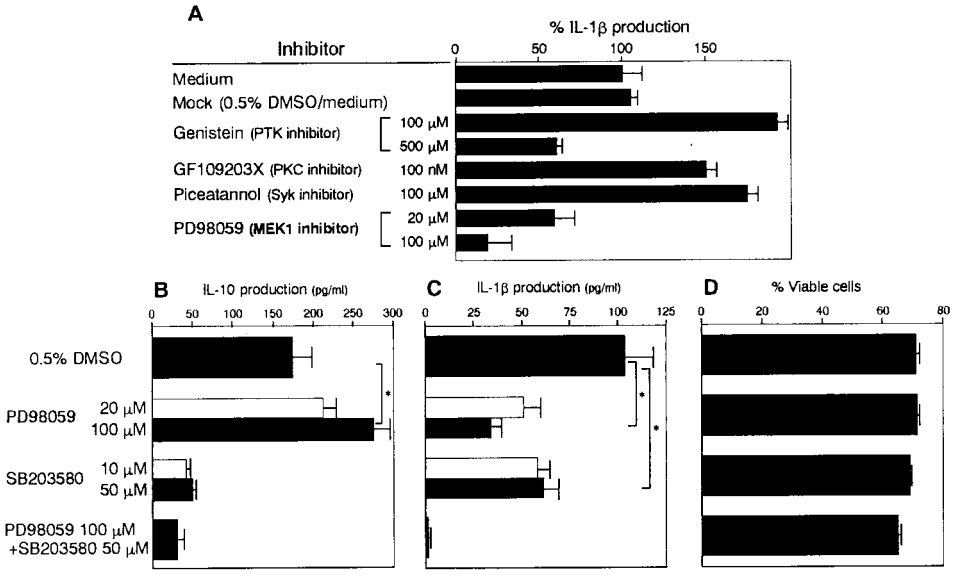

FIGURE 4. Effect of PD98059 and SB203580 on IL-10 and IL-1β secretion. *A–C,* Peptide-pulsed monocytes were precultured for 60 min with the indicated inhibitors, at the indicated concentrations, and cocultured with emetine-treated T cells (BC20.7) for 24 h. Culture supernatants were collected and stored in aliquots at −80°C until determinations of cytokine concentrations. One hundred percent IL-1β production was 115 pg/ml. *D,* Cell viability (for the highest concentrations of the inhibitors shown in *B* and *C*) was determined by trypan blue exclusion. Results were expressed as means ± SD of triplicate cultures. *, $p < 0.05$.

MEK-1 inhibitor PD98059 inhibited IL-1β secretion in a dose-dependent manner, whereas the PKC inhibitor GF109203X (IC_{50}, 20 nM; Ref. 23) or protein tyrosine kinase (PTK) inhibitors did not clearly do so. Genistein exhibited a biphasic effect and inhibited IL-1β production at high concentrations (500 μM), which corroborates the results shown in Fig. 2*A*. We next examined the effect of inhibitors specific for individual MAP kinase(s) on the production of IL-1β and IL-10 using emetine-treated BC20.7 T cells and peptide-pulsed monocytes. Interestingly, the 50 μM p38 inhibitor SB203580 (24) markedly inhibited IL-10 production, whereas PD98059 rather enhanced IL-10 production (Fig. 4*B*). On the other hand, both PD98059 and SB203580 only partially inhibited IL-1β (Fig. 4*C*). Moreover, a combination of PD98059 and SB203580 completely abrogated IL-1β. Because these inhibitors did not decrease cell viability after 24-h culture (Fig. 4*D*), it is likely that the kinase inhibitors at the concentration we used exerted specific inhibitory effects on monocytes. When DQ- and DP-restricted T cells were used for stimulation, similar effects of PD98059 and SB203580 on monokine secretion were observed, except that the enhancing effect of PD98059 on IL-10 was only marginal (data not shown). These observations suggest that 1) p38 is involved in both IL-1β and IL-10 production induced by ligating DR molecules expressed on monocytes; 2) the MEK-1-Erk pathway is only partially involved in IL-1β production, being independent from p38-associated IL-1β production; and 3) activation of Erk may inhibit p38-mediated IL-10 production (Fig. 5).

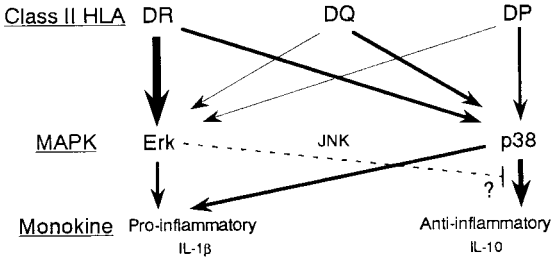

FIGURE 5. Summary of class II HLA-mediated MAP kinase (MAPK) activation. Note that other class II signaling elements that can be additive or modify the signaling via MAP kinases are not illustrated.

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. We then examined the production of IFN-γ and IL-4 using *Der f*-specific T cell lines. As shown in Fig. 6*A*, DR-

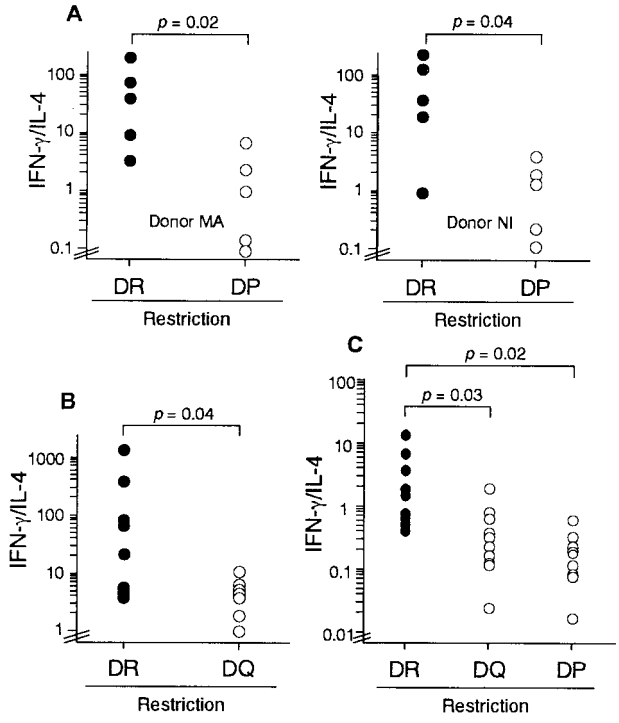

FIGURE 6. Restriction molecules and cytokine production patterns of short-term T cell lines. *Der f* (crude mite Ag)-specific short-term T cell lines (*A*), PPD-specific short-term T cell lines (*B*), and X19-reactive T cell clones (*C*) of various restriction patterns were restimulated with excess concentrations of Ags (*A* and *B*, 10 μg/ml; *C*, 500 μM) and then after a 48-h incubation, culture supernatants were collected for measurements of IFN-γ and IL-4 production by ELISA. One spot indicates one cell line.

restricted T cell lines produced more IFN-γ than IL-4, but DP-restricted T cell lines produced more IL-4 than IFN-γ ($p = 0.02$ and 0.04 in donors MA and NI, respectively). mAbs used in this study did not induce monokine secretion when used as a soluble form (data 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*010-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. 6*B*). DR-restricted T cell lines produced more IFN-γ than IL-4, but DQ-restricted lines produced more IL-4 than IFN-γ ($p = 0.04$ in donor MA). We then used X19 (random 19-mer peptide) to confirm the phenomenon observed in earlier studies because 1) DQ-restricted/*Der f*-reactive and DP-restricted/PPD-reactive T cells were not readily established and 2) X19 can stimulate most $CD4^+$ memory T cells to proliferate in the presence of cytokines under cloned conditions.[4] Indeed, DR-, DQ-, and DP-restricted T cell lines were obtained, the cytokine profiles of which again exhibited similar results (Fig. 6*C*). When we titrated down the peptide concentration for DR-restricted responses, we found that lower concentrations of X19 peptide did not lead to DQ-/DP-restricted patterns of cytokines (data not shown), which was indeed the case when emetine-treated BC20.7 T cells were incubated with monocytes in the presence of lower concentrations of the antigenic peptide (data not shown). These data indicate that DR-restricted and already activated peripheral $CD4^+$ T cells carry the Th1-prone phenotype compared with DQ-/DP-restricted T cells, albeit the segregation pattern being incomplete.

Discussion

Monokines contribute to T cell response patterns, both at differentiation (25, 26) and effector stages (27, 28). Differential activation of monokines through HLA-DR, DQ, and DP molecules described in this study may indicate distinct monocyte activation patterns, depending on restriction molecules in Ag presentation. Therefore, the diversity of class II HLA molecules (such as DR, DQ, and DP) should increase not only the variety of binding peptides, but also the variety of immune response patterns. Thus, the need for generation of a multigene family of class II MHC seems apparent. Cytokine imbalances among T cell clones or distinct expression of membrane molecules such as CDw150 (29), are unlikely to be major factors for the current observation because a similar phenomenon was observed when mAbs were used for purposes of stimulation. Recent studies by Viola et al. (30) demonstrated that mAbs immobilized on beads deliver more physiological signals than do solid-phase mAbs. Indeed, when L243, 1a3, and B7/21 were immobilized on beads, they exhibited similar results; i.e., L243 beads stimulated monocytes to phosphorylate Erk and produce a higher IL-1β:IL-10 ratio than did 1a3 or B7/21 (data not shown). Soluble anti-class II induced only marginal signaling (data not shown), unlike many other studies using B cells. This is probably due to lower class II expression levels on monocytes compared with B cells. When T cells were preactivated for 16 h with peptide-pulsed monocytes, treated with emetine, and subjected to coculture with freshly isolated monocytes, the IL-1β:IL-10 ratio remained unchanged and levels of IL-1β and IL-10 production became even lower (data not shown), indicating that 1) expression of cell surface molecules induced as a result of TCR-peptide-MHC interaction contribute little to IL-1β/IL-10 and 2) decreased monokine production induced by activated T cells may reflect the TCR down-modulation induced by activation. Indeed, human T cell clones used in this study were stimulated with peptides every 6–7 days and CD154 did not return to null expression even on day 7 (1), which should have contributed to IL-12 production.

The observation that IFN-γ/IL-4 produced by T cells is associated with HLA-restriction molecules even in freshly isolated short-term T cell clonal responses to crude protein Ags or randomized peptide Ags is evidence that the phenomenon is not limited to three T cell clones used in this study. In other words, while not being complete as evidenced by Fig. 6, DR-restricted Th2 or DQ-/DP-restricted Th1 can exist, HLA class II subregions may determine T cell differentiation patterns or IFN-γ/IL-4, probably through monocyte responses. However, one can speculate that the DR-peptide complex delivers the strongest avidity between TCR, leading to Th1-prone responses (31, 32). In this regard, absence of DR-restricted T cell clones with low IFN-γ/IL-4 as shown in Fig. 6 rules out this possibility, because low-affinity DR-binding peptides should exist and would activate Th2-prone responses, if the phenomenon is attributed to avidity alone. Indeed, it is likely that such a phenomenon is attributed to high IL-12 production through DR signaling (Table I). In activating naive T cells to generate primed Th1/Th2, dendritic cells should play pivotal roles (33) and class II-mediated signaling in dendritic cells awaits to be determined.

We also examined the effect of peptide concentrations on the IL-10:IL-1β ratio using T cell clones and peptide-pulsed monocytes. Indeed, emetine-treated BC20.7 T cells (DR-restricted) stimulated peptide-pulsed monocytes to produce IL-10 and IL-1β at practically a constant ratio when monocytes were pulsed with lower concentrations of the peptides (data not shown), thus indicating that weaker HLA-peptide-TCR interaction does not alter the pattern of monocyte responses to secrete monokines. It is also known that 1) different peptide sequences are not required to differentially drive Th1/Th2 directions by class II (34) and 2) differential signaling through TCR (but not through monokine receptors) can be induced depending on the peptide concentrations used, which may lead to differential activation of Th1/Th2 (35, 36). Therefore, restriction molecule (DR, DQ, or DP) is not the only one but at least one of the important molecules to determine monocyte responses. Another possibility to be ruled out is the effect of class II expression levels. This question can be addressed using class II transfection into human class II-deficient monocytic cells because class II^{high} and class II^{low} populations of peripheral monocytes/macrophages may belong to distinct cell lineages (28), and stimulation of monocytes by IFN-γ leads to PTK and PKC activation and hampers natural signaling processes through class II (37).

All three MAP kinases phosphorylate substrates on serine/threonine residues. Erks are characteristically activated by growth factors, whereas JNK/stress-activated protein kinase and p38 kinase are strongly activated by UV irradiation, osmotic stress, and inflammatory cytokines (38). Thus, they are differentially activated by stimuli other than class II HLA isoforms. In other words, ligation of DR, DQ, and DP is another set of stimuli that differentially activates MAP kinases. In other experimental systems (24), where monocytes were stimulated with LPS, p38 and Erk but not JNK was activated, as observed in the present study. Interestingly, inhibition of p38 suppressed both IL-10 and IL-1β, whereas that of Erk partially suppressed IL-1β but not IL-10 (24), which corroborates results we obtained. Although Erk was markedly phosphorylated by anti-DR Ab, the involvement in monokine secretion was only partial. Other consequences, such as proliferation, might be a major outcome of Erk activation through DR; however, proliferation was not readily detected using monocytes. Another set of experiments using fibroblasts is underway to delineate the biological significance of DR-mediated Erk activation. As shown in Figs. 2 and 4, the effect of genistein on IL-1β production was biphasic. The enhancing effect at 100 μM may indicate that certain PTKs have inhibitory effects on the IL-1β production induced by DR ligation. On the contrary, it is likely that the suppressive effect at 500 μM reflects the effect on PTKs whereby the ras-raf pathway is activated.

Syk is associated with Erk (39) and involved in signaling through MHC molecules (12, 40). We observed a slight enhancement of monokine production by PKC inhibitor GF109203X and Syk inhibitor piceatannol (Figs. 2 and 4). The precise mechanisms are yet to be determined, but one may speculate that they negatively regulate monokine production induced by ligating class II HLA.

Because transmembrane and intracellular domains are markedly different among α- and β-chains of HLA-DR, -DQ, and -DP and MHC molecules have no immunoreceptor tyrosine-based activation motif, it seems reasonable to speculate that HLA-DR, -DQ, or -DP molecules are associated with distinct signal transduction molecules. Differential endosomal trafficking/recycling (41), differential signaling in monocyte subsets (28), including contaminated dendritic cells, and even differential localization in membrane microdomains (42, 43) would also need to be considered. Studies currently underway will address these questions using various monocytic cell lines and mass mapping techniques.

Acknowledgments

We thank M. Ohara for readings of this manuscript.

References

1. Matsuoka, T., H. Kohrogi, M. Ando, Y. Nishimura, and S. Matsushita. 1996. Altered TCR ligands affect antigen-presenting cell responses: up-regulation of IL-12 by an analogue peptide. *J. Immunol. 157:4837.*
2. Cambier, J. C., M. K. Newell, L. B. Justement, J. C. McGuire, K. L. Leach, and Z. Z. Chen. 1987. Ia binding ligands and cAMP stimulate nuclear translocation of PKC in B lymphocytes. *Nature 327:629.*
3. Lane, P. J., F. M. McConnell, G. L. Schieven, E. A. Clark, and J. A. Ledbetter. 1990. The role of class II molecules in human B cell activation: association with phosphatidyl inositol turnover, protein tyrosine phosphorylation, and proliferation. *J. Immunol. 144:3684.*
4. Mooney, N. A., C. Grillot-Courvalin, C. Hivroz, L. Y. Ju, and D. Charron. 1990. Early biochemical events after MHC class II-mediated signaling on human B lymphocytes. *J. Immunol. 145:2070.*
5. Bishop, G. A. 1991. Requirements of class II-mediated B cell differentiation for class II cross-linking and cyclic AMP. *J. Immunol. 147:1107.*
6. Kansas, G. S., and T. F. Tedder. 1991. Transmembrane signals generated through MHC class II, CD19, CD20, CD39, and CD40 antigens induce LFA-1-dependent and independent adhesion in human B cells through a tyrosine kinase-dependent pathway. *J. Immunol. 147:4094.*
7. Truman, J. P., M. L. Ericson, C. J. Choqueux-Seebold, D. J. Charron, and N. A. Mooney. 1994. Lymphocyte programmed cell death is mediated via HLA class II DR. *Int. Immunol. 6:887.*
8. Nabavi, N., G. J. Freeman, A. Gault, D. Godfrey, L. M. Nadler, and L. H. Glimcher. 1992. Signalling through the MHC class II cytoplasmic domain is required for antigen presentation and induces B7 expression. *Nature 360:266.*
9. Harton, J. A., A. E. Van Hagen, and G. A. Bishop. 1995. The cytoplasmic and transmembrane domains of MHC class II beta chains deliver distinct signals required for MHC class II-mediated B cell activation. *Immunity 3:349.*
10. Rich, T., S. E. Lawler, J. M. Lord, V. M. Blancheteau, D. J. Charron, and N. A. Mooney. 1997. HLA class II-induced translocation of PKC alpha and PKC beta II isoforms is abrogated following truncation of DR beta cytoplasmic domains. *J. Immunol. 159:3792.*
11. Morio, T., R. S. Geha, and T. A. Chatila. 1994. Engagement of MHC class II molecules by staphylococcal superantigens activates src-type protein tyrosine kinases. *Eur. J. Immunol. 24:651.*
12. Kanner, S. B., L. S. Grosmaire, J. Blake, G. L. Schieven, S. Masewicz, N. Odum, and J. A. Ledbetter. 1995. ZAP-70 and p72 syk are signaling response elements through MHC class II molecules. *Tissue Antigens 46:145.*
13. Mehindate, K., J. Thibodeau, M. Dohlsten, T. Kalland, R. P. Sekaly, and W. Mourad. 1995. Cross-linking of major histocompatibility complex class II molecules by staphylococcal enterotoxin A superantigen is a requirement for inflammatory cytokine gene expression. *J. Exp. Med. 182:1573.*
14. Hirayama, K., S. Matsushita, I. Kikuchi, M. Iuchi, N. Ohta, and T. Sasazuki. 1987. HLA-DQ is epistatic to HLA-DR in controlling the immune response to schistosomal antigen in humans. *Nature 327:426.*
15. Ikezawa, Z., Z. A. Nagy, and J. Klein. 1984. Manipulation of anti-LDH-B response by T suppressor factors. *J. Immunol. 132:1605.*
16. Matsushita, S., H. Kohsaka, and Y. Nishimura. 1997. Evidence for self and non-self peptide partial agonist that prolong clonal survival of mature T cells in vitro. *J. Immunol. 158:5685.*
17. Fujita, H., S. Senju, H. Yokomizo, H. Saya, M. Ogawa, S. Matsushita, and Y. Nishimura. 1998. Evidence that HLA class II-restricted human CD4$^+$ T cells specific to p53 self peptides respond to p53 proteins of both wild and mutant forms. *Eur. J. Immunol. 28:305.*
18. Schmid-Alliana, A., L. Menou, S. Manie, H. Schmid-Antomarchi, M. A. Millet, S. Giuriato, B. Ferrua, and B. Rossi. 1998. Microtubule integrity regulates Src-like and extracellular signal-regulated kinase activities in human pro-monocytic cells. *J. Biol. Chem. 273:3394.*
19. Weaver, C. T., and E. R. Unanue. 1986. T cell induction of membrane IL-1 on macrophage. *J. Immunol. 137:3868.*
20. Parry, S. L., M. Sebbag, M. Feldmann, and F. M. Brennan. 1997. Contact with T cells modulates monocyte IL-10 production: role of T cell membrane TNF-alpha. *J. Immunol. 158:3673.*
21. Yoshimoto, T., H. Nagase, T. Ishida, J. Inoue, and H. Nariuchi. 1997. Induction of interleukin-12 p40 transcript by CD40 ligation via activation of nuclear factor-κB. *Eur. J. Immunol. 27:3461.*
22. Becherel, P. A., L. LeGoff, C. Frances, O. Chosidow, J. J. Guillosson, P. Debre, M. D. Mossalayi, and M. Arock. 1997. Induction of IL-10 synthesis by human keratinocytes through CD23 ligation: a cyclic adenosine 3′,5′-monophosphate-dependent mechanism. *J. Immunol.* 159:5761.
23. Toullec, D., P. Pianetti, H. Coste, P. Bellevergue, T. Grand-Perret, M. Ajakane, V. Baudet, P. Boissin, E. Boursier, F. Loriolle, et al. 1991. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem. 266:15771.*
24. Foey, A. D., S. L. Parry, L. M. Williams, M. Feldmann, B. M. Foxwell, and F. M. Brennan. 1998. Regulation of monocyte IL-10 synthesis by endogenous IL-1 and TNF-α: role of the p38 and p42/44 mitogen-activated protein kinases. *J. Immunol. 160:920.*
25. Hsieh, C. S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy. 1993. Development of Th1 CD4$^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science 260:547.*
26. Iwasaki, A., and B. L. Kelsall. 1999. Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. *J. Exp. Med. 190:229.*
27. Gately, M. K., L. M. Renzetti, J. Magram, A. S. Stern, L. Adorini, U. Gubler, and D. H. Presky. 1998. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. *Annu. Rev. Immunol. 16:495.*
28. Loercher, A. E., M. A. Nash, J. J. Kavanagh, C. D. Platsoucas, and R. S. Freedman. 1999. Identification of an IL-10-producing HLA-DR-negative monocyte subset in the malignant ascites of patients with ovarian carcinoma that inhibits cytokine protein expression and proliferation of autologous T cells. *J. Immunol. 163:6251.*
29. Isomaki, P., G. Aversa, B. G. Cocks, R. Luukkainen, R. Saario, P. Toivanen, J. E. de Vries, and J. Punnonen. 1997. Increased expression of signaling lymphocytic activation molecule in patients with rheumatoid arthritis and its role in the regulation of cytokine production in rheumatoid synovium. *J. Immunol. 159:2986.*
30. Viola, A., S. Schroeder, Y. Sakakibara, and A. Lanzavecchia. 1999. T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science 283:680.*
31. Constant, S., C. Pfeiffer, T. Pasqualini, and K. Bottomly. 1995. Extent of TCR ligation can determine the functional differentiation of native CD4$^+$ T cells. *J. Exp. Med. 182:1591.*
32. Hosken, N. A., K. Shibuya, A. W. Heath, K. M. Murphy, and A. O'Garra. 1995. The effect of antigen dose on CD4$^+$ T helper cell phenotype development in a TCR-$\alpha\beta$-transgenic model. *J. Exp. Med. 182:1579.*
33. Inaba, K., J. P. Metlay, M. T. Crowley, and R. M. Steinman. 1990. Dendritic cells pulsed with protein antigens in vitro can prime antigen-specific, MHC-restricted T cells in situ. *J. Exp. Med. 172:631.*
34. Pfeiffer, C., J. Stein, S. Southwood, H. Ketelaar, A. Sette, and K. Bottomly. 1995. Altered peptide ligands can control CD4 T lymphocyte differentiation in vivo. *J. Exp. Med. 181:1569.*
35. Constant, S. L., and K. Bottomly. 1997. Induction of Th1 and Th2 CD4$^+$ T cell responses: the alternative approaches. *Annu. Rev. Immunol. 15:297.*
36. Kersh, E. N., A. S. Shaw, and P. M. Allen. 1998. Fidelity of T cell activation through multistep T cell receptor ζ phosphorylation. *Science 281:572.*
37. Lee, Y.-J., R. B. Panek, M. Huston, and E. N. Benveniste. 1995. Role of protein kinase C and tyrosine kinase activity in IFN-γ-induced expression of the class II MHC gene. *Am. J. Physiol. 268:C127.*
38. Jun, C.-D., C. D. Oh, H. J. Kwak, H. O. Pae, J. C. Yoo, B. M. Choi, J. S. Chun, R. K. Park, and H. T. Chung. 1999. Overexpression of protein kinase C isoforms protects RAW 264.7 macrophages from nitric oxide-induced apoptosis: involvement of c-Jun N-terminal kinase/stress-activated protein kinase, p38 kinase, and CPP-32 protease pathways. *J. Immunol. 162:3395.*
39. Xu, R., R. Seger, and I. Pecht. 1999. Extracellular signal-regulated kinase activates Syk: a new potential feedback regulation of Fcε receptor signaling. *J. Immunol. 163:1110.*
40. Tabata, H., T. Matsuoka, F. Endo, Y. Nishimura, and S. Matsushita. 2000. Ligation of HLA-DR molecules on B cells induces enhanced expression of IgM heavy chain genes in association with Syk activation. *J. Biol. Chem. 275:34998.*
41. Laufer, T. M., S. T. Smiley, A. Ranger, V. K. Clements, S. Ostrand-Rosenberg, and L. H. Glimcher. 1997. Single amino acid mutations in the murine MHC class II A β cytoplasmic domain abrogate antigen presentation. *J. Immunol. 159:5914.*
42. Vereb, G., J. Matko, G. Vamosi, S. M. Ibrahim, E. Magyar, S. Varga, J. Szoellosi, A. Jenei, R. Gaspar, Jr., T. A. Waldmann, and S. Damjanovich. 2000. Cholesterol-dependent clustering of IL-2Rα and its colocalization with HLA and CD48 on T lymphoma cells suggest their functional association with lipid rafts. *Proc. Natl. Acad. Sci. USA 97:6013.*
43. Anderson, H. A., E. M. Hiltbold, and P. A. Roche. 2000. Concentration of MHC class II molecules in lipid rafts facilitates antigen presentation. *Nat. Immunol. 1:156.*