Cross-linking HLA-DR molecules on Th1 cells induces anergy in association with increased level of cyclin-dependent kinase inhibitor p27Kip1

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Abstract

HLA class II molecules play pivotal roles in antigen presentation to CD4+ T cells. We investigated signaling via HLA-DR molecules expressed on CD4+ T cells. 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. Whereas cross-linking of HLA-DR and CD3 resulted in secretion of the same levels of IFN-γ and IL-8, secretion of IL-10 induced by cross-linking of HLA-DR was less than that induced by cross-linking of CD3 on CD4+ T cells. Interestingly, expression of p27Kip1 but not p21Cip1 increased after stimulation by either anti-HLA-DR or anti-CD3 mAb. This was indeed the case, when T cells were rendered anergic using a soluble form of antigenic peptide. In contrast, T cells stimulated by peptide-pulsed PBMC expressed little p27Kip1. We propose that signaling via HLA-DR molecules on CD4+ T cells at least in part contributes to the induction of T cell anergy, through the upregulated expression of the p27Kip1. The implication of our finding is that HLA-DR molecules play a role in human T cell anergy induced by a soluble form of antigenic peptide. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

The important role of HLA class II molecules is antigen presentation to CD4+ T cells. In recent years, it has been reported that signals are transmitted to antigen presenting cells (APC) via HLA class II molecules when APCs present antigens to T cells. We earlier reported that interactions between a CD4+ T cell clone and monocytes 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)

Abbreviations: Ab, antibody; APC, antigen presenting cell; BCGa, Bacillus Calmette-Guérin a; cAMP, cyclic AMP; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence activated cell sorter; FITC, fluorescein isothiocyanate; Fmoc, 9-fluorenylmethoxycarbonyl; HLA, human histocompatibility leukocyte antigen; IFN-γ, interferon-γ; IL, interleukin; IL-2R, interleukin-2 receptor; mAb, monoclonal antibody; MHC, major histocompatibility complex; PAGE, polyacrylamide gel electrophoresis; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PTK, protein tyrosine kinase; rIL-2, recombinant interleukin-2; SDS, sodium dodecyl sulfate; TCR, T cell receptor; Th1, T helper type 1.

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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 monokines and chemokines from monocytes, cross-linking HLA-DR and -DP but not -DQ molecules expressed on T cells by immobilized mAb cells. Cross-linking HLA-DR and -DP but not -DQ molecules in (MAPK) [14], and in B cells, signals via HLA class II molecules influence IgM expression through activation of Syk [15].

Activated T cells of humans and of most rodents but not of mice, express MHC class II molecules, which also play pivotal roles in signal transduction into T cells. Cross-linking HLA-DR and -DP but not -DQ molecules expressed on T cells by immobilized mAb was reported to enhance proliferative T cell responses to IL-2 [16]. This phenomenon was at least partly mediated by an up-regulation of the high-affinity IL-2 receptor [16]. Cross-linking HLA-DR molecules expressed on T cells induced tyrosine phosphorylation of several proteins, including PLC-γ1, and elevation of cytoplasmic free calcium [Ca2+]i, [17–21]. Protein tyrosine kinase (PTK) activation was obligatory for these class II signals [16,18,20]. Another study by Kanner et al. [12] demonstrated that ligation of class II activates Syk in B cells. Moreover, engagement of class II molecules on the THP-1 monocyte cell line with staphylococcal enterotoxin A induced IL-1 /p97 and TNF-/p103 production [13]. In our recent previous studies, signals via HLA class II molecules influence productions of monokines and chemokines from monocytes, through activation of mitogen-activated protein kinase (MAPK) [14], and in B cells, signals via HLA class II molecules influence IgM expression through activation of Syk [15].

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2. Materials and methods

2.1. Reagents

L243 (anti-HLA-DRB1 + DRB4 IgG2a, monomorphic; Ref. [26]) was purified from the ascites-form of mAbs, using a Protein A column (Pierce, Rockford, IL). Mouse IgG and mouse IgG2a were purchased from BioPur AG (Bubendorf, Switzerland) and Biogenesis (Poole, UK), respectively. L243 and control mouse IgG were conjugated with biotin (ImmuNoPure NHS- LC-Biotin, Pierce). Anti-p27Kip1 Ab (sc-528), anti-p21Cip1 Ab (sc-397), anti-p16INK4a Ab (sc-468) and anti-p15INK4b Ab (sc-612) were purchased from Santa Cruz Biotechnology, Inc. Bacillus Calmette-Guérin (BCG) a p84-100 [27] was synthesized, using a solid-phase simultaneous multiple peptide synthesizer PSSM-8 (Shimadzu Corp., Kyoto, Japan), based on the F-moc (9-fluorenylmethoxycarbonyl) strategy and using a 10-fold molar excess of single F-moc amino acids, followed by purification using C18 reverse-phase high performance liquid chromatography (Millipore, Waters, Milford, MA).

2.2. Human T cell clone SF36.16

Human CD4+ T cell clone SF36.16 that recognizes DRB1*0405 + residues 84-100 of BCGa protein (BCGap84-100; EEYLILSARDVLAVVSK) was as described [28]. SF36.16 produced 40.5 ng/ml of interferon-γ (IFN-γ) and 102 pg/ml of IL-4, at plateau responses, thus classified as Th1/Th0 [28]. SF36.16 cells were fed weekly with 50 U/ml human rIL-2 in the presence of irradiated (4500 cGy) DRB1*0405-positive PBMC prepulsed with BCGap84-100 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.

2.3. Cell surface Ag analysis

Cultured cells were stained with FITC-conjugated anti-CD4 mAb (PharMingen, San Diego, CA), FITC-
conjugated anti-HLA-DR mAb (PharMingen, San Diego, CA) and Propidium Iodide (PI) (Sigma, St. Louis, MO) on days 0, 6, 7 and 8. Stained cells were analyzed using a FACScan flow cytometer (Becton Dickinson, San Diego, CA). Viable cells were gated in PI staining. Data analysis was performed using Cell Quest Software (Becton Dickinson, San Diego, CA).

2.4. Stimulation of SF36.16 T cells

Twenty micrograms per milliliter anti-DR mAb (L243) and control mouse IgG2a as well as 5 μg/ml anti-CD3 mAb (OKT3) were precoated onto 96- or 24-well flat-bottom culture plates for 2 days. After extensive washing of the plates with PBS, T cells were added and incubation was carried out at 37 °C in a CO2 incubator. Culture supernatants, collected 24 h after initiation of the culture were stored in aliquots at −80 °C until determinations of lymphokine concentrations. To examine kinetics of proliferation induced by cross-linking HLA-DR or CD3 molecules on CD4+ T cells, CD4+ T cells were cultured for 0, 24, 48, 72, 120 or 168 h, followed by culture in the presence of 1 Ci/well [3H]thymidine during the final 16-h period. The incorporated radioactivity was measured by liquid scintillation counting. When harvesting the cells, 0.1 N NaOH was added to the wells to detouch cells from the plates.

2.5. 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 preincubated in the presence of 1 μCi/well [3H]thymidine during the final 16-h period. The incorporated radioactivity was measured by liquid scintillation counting. When harvesting the cells, 0.1 N NaOH was added to the wells to detouch cells from the plates.

2.6. ELISAs

The human IFN-γ and IL-10 ELISA kits (BioSource International, Camarillo, CA) and human IL-8 ELISA kit (Central Laboratory of the Netherlands Red Cross, Amsterdam, The Netherlands) were used for quantitation of lymphokines in the supernatants, according to manufacturer’s instructions.

2.7. Western immunoblot analysis

T cells were added to 24-well culture plates in which anti-class II HLA-DR mAb (L243) or anti-CD3 (OKT3) mAb had been immobilized. After a 7-day culture at 37 °C, T cells were washed twice with RPMI 1640, followed by lysing using lysing buffer (150 mM NaCl, 20 mM Tris, pH 7.6, 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 a nitrocellulose membrane. After blocking with 10% skim milk and 0.1% Tween 20 in TBS, the membrane was incubated with Abs specific for CDK inhibitors p15INK4b, p16INK4a, p21Cip1 and p27Kip1, washed extensively with 0.1% Tween 20 in TBS, and subjected to chemiluminescence detection with peroxidase-conjugated anti-mouse IgG Ab using an ECL kit (Amersham, UK).

3. Results

3.1. Kinetics of CD4 and HLA-DR expression

Cloned T cells (SF36.16) were co-cultured with peptide-pulsed and irradiated PBMC on day 0. We examined the proportion of CD4+ cells in the culture on days 0, 6, 7 and 8, by using FACS (Fig. 1). Double staining was done using PI and FITC, then based on the PI staining (data not shown), viable cells (> 85%) was gated for analyses. Many CD4+ cells on day 0 consisted mainly of macrophages. B cells and CD4− T cells. However, on days 6, 7 and 8, CD4+ cells occupied the majority of cultured cells (98.8, 98.7 and 99.1%, respectively). Therefore, it seems unlikely that the effect of HLA-DR molecules expressed on macrophages and B cells are significant when cultured cells on days 6, 7 and 8 are used.

We then examined expression of HLA-DR molecules on cultured cells on days 6, 7 and 8 (Fig. 1), by double staining, as described above. Although, cultured cells gradually decreased their HLA-DR expression, they indeed expressed HLA-DR molecules on days 6, 7 and 8. Therefore, we use SF36.16 on day 6, for subsequent experiments.

3.2. 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. 2). 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 h, 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.

3.3. 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. 3A). Thus, when these cells are re-stimulated with 1 nM antigenic peptide and irradiated autologous PBMC, there may be 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 autologous PBMC (Fig. 3A).

SF36.16 cells cultured in the presence of solid-phase anti-CD3 mAb OKT3 for 7 days also showed low responsiveness (Fig. 3B). 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. 3C). 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. 3D). 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. 3D).

Fig. 2. 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 h), 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.

Fig. 1. Kinetics of CD4 and HLA-DR expression. Cloned T cells (SF36.16; 0.1–0.5 x 10^6 cells per well) were co-cultured with peptide-pulsed and irradiated PBMC (1.5 x 10^6 cells per well) on day 0, and the proportion of CD4+ cells in cultures on days 0, 6, 7 and 8 was examined, using FACS. Double staining was done with PI and FITC, and viable cells was gated (population > 85%), and analyzed after FITC staining. Histograms obtained by anti-CD4/anti-DR and an isotype-matched control Ab are shown by solid lines and dotted lines, respectively.
3.4. Cytokine production of T cells induced by cross-linking CD3 or HLA-DR molecules

As up-regulation of IL-10 induces T cell clonal anergy, we examined the cytokine production of T cells induced by cross-linking CD3 or HLA-DR molecules. When HLA-DR and CD3 molecules on CD4+ T cells were cross-linked by solid-phase mAbs, the same levels of IFN-γ and IL-8 were secreted (Fig. 4). However, secretion of IL-10 induced by cross-linking of HLA-DR was only half that induced by cross-linking of CD3 on CD4+ T cells.

3.5. Expression of CDK inhibitors p21Cip1 and p27Kip1 in anergic CD4+ T cells

As shown in Fig. 5, T cells with clonal anergy induced by stimulation with the soluble form of an antigenic peptide, expressed higher level of CDK inhibitor p27Kip1 on day 7, than did those cultured with irradiated autologous PBMC prepulsed with 5 μM agonistic peptide. Likewise, anergic T cells induced by cross-linking it’s CD3 or HLA-DR, expressed higher level of CDK inhibitor p27Kip1. In contrast, both expressed the same levels of CDK inhibitor p21Cip1 (Fig. 5), p15INK4b and p16INK4a (data not shown). Neither T cells stimulated with control IgG (Fig. 2) 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.

4. Discussion

We obtained evidence that cross-linking of HLA-DR molecules expressed on cloned Th1 cells induced prolif-
This is the first report that cross-linking HLA-DR expressed on activated T cells alone induces T cell clonal anergy. The anti-DR-induced IL-10 was less than 100 pg/ml, a value which did not exceed that induced by anti-CD3. Indeed, neutralizing Ab to IL-10 did not abrogate the induction of anergy induced by anti-DR Ab (data not shown). This is evidence that secretion of a humoral factor such as IL-10 is not essential for the induction of anergy induced by anti-HLA-DR Ab. T cells anergized by anti-HLA-DR, did proliferate in the presence of exogenous IL-2 during the re-stimulation phase, thus indicating that there is no defect in signaling through the IL-2R, unlike the anergy induced by staphylococcal enterotoxin A [37]. Because staphylococcal enterotoxin A cross-links not only HLA-DR but also DQ and DP, and signaling through HLA-DR, -DQ and -DP differentially activate MAPK [14], it might be that staphylococcal enterotoxin A and anti-DR induce distinct signaling events.

Similarity between anti-CD3- and anti-DR-induced signaling events has been reported. Kanner et al. reported that signaling via MHC class II activates ZAP-70-dependent activation of phospholipase C [12], resulting in phosphoinositot turn-over and Ca\textsuperscript{2+} flux [20]. However, the signaling pathways coupled with MHC class II may be distinct from those coupled with TCR/CD3, because: (1) engagement of the transmembrane phosphatase CD45 inhibited Ca\textsuperscript{2+} fluxes triggered via TCR/CD3 but not those triggered via MHC class II; and (2) IL-2 secretion was induced by signaling via TCR/CD3 but not by signaling via MHC class II [20].

The anergy induced by soluble antigenic peptide was associated, not with increased expression of p21\textsuperscript{Cip1} and p15\textsuperscript{INK4B} and p16\textsuperscript{INK4A} but rather with that of p27\textsuperscript{Kip1}, which corroborates well with previous reports by others [23,24]. The p27\textsuperscript{Kip1} reportedly binds cyclin D-CDK4, 6 and cyclin E-CDK2 complexes, and leads to G1 arrest by inhibiting those molecules. It seems reasonable to consider that sustained up-regulation of p27\textsuperscript{Kip1} plays pivotal roles in the induction of T cell anergy.

In case of the anergy induced by soluble antigenic peptide, signals should be transmitted to T cells not only via TCR but also via class II HLA because T cells express both TCR and HLA-DR. Although, anergy induced by the lack of co-stimulation was rescued by signaling via CD28, anergy induced by soluble antigenic peptide was not rescued [22], which is also the case in our present study (data not shown). It is therefore likely that molecular mechanisms differ between anergy induced by the lack of co-stimulation and that induced by anti-DR, even though the behavior of CDK inhibitors are apparently the same.
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