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MHC class II structural requirements for the association with Igα/β, and signaling of calcium mobilization and cell death

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Abstract

Emerging evidence indicates that in addition to their well-characterized role in antigen presentation, MHC II molecules transmit signals that induce death of APCs. Appropriately timed APC death is important for prevention of autoimmunity. Though the exact mechanism of MHC II-mediated cell death signaling is unknown, the response appears independent of caspase activation and does not involve Fas–FasL interaction. Here we investigated MHC II structural requirements for mediation of cell death signaling in a murine B cell lymphoma. We found that neither the transmembrane spanning regions nor the cytoplasmic tails of MHC II, which are required for MHC II-mediated cAMP production and PKC activation, are required for the death response. However, mutations in the connecting peptide region of MHC II α chain (αCP), but not the β chain (βCP), resulted in significant impairment of the death response. The αCP mutant was also unable to mediate calcium mobilization responses, and did not associate with Igα/β. Knock-down of Igβ by shRNA eliminated the MHC II-mediated calcium response but not cell death. We propose that MHC II mediates cell death signaling via association with an undefined cell surface protein(s), whose interaction is partially dependent on αCP region.

Keywords: B cells; MHC class II; Apoptosis; Signal transduction; Igα/β

1. Introduction

MHC II molecules function in presentation of antigenic peptides to $CD4^+$ T cells leading to mutual cell activation and propagation of the humoral immune response. Several lines of evidence suggest that MHC class II also transduce signals for B cell responses to thymus-dependent antigens. These include the observations that in the context of external signals, aggregation of MHC class II on activated B cells *in vitro* induces biologic responses associated with humoral immune responses *in vivo* including, immunoglobulin isotype switching, antibody secretion, cytoskeletal reorganization and proliferation [1–3].

MHC class II signaling can also lead to cell death via an apoptotic mechanism [4]. It has been proposed that MHC II-mediated cell death may play a role in eliminating those APC that have already presented their antigens to T cells thereby terminating immune response [5]. Consistent with the hypothesis is the recent finding that prolongation of dendritic cells survival can lead to autoimmunity [6].

The ability of MHC II to mediate cell death signals has led to investigation of the potential of MHC II as a therapeutic target [7]. MHC II mAbs induced rapid and potent cell death in activated MHC II^+ cells [7]. Two anti-human MHC II mAbs apolizumab (Remitogen) and Lym-1 (Oncolym) are currently in clinical trial [8,9]. Meanwhile, fully humanized MHC class II mAbs have been generated and tested in lymphoma/leukemia patient samples and primates with promising results [10].

Abbreviations: PS, phosphatidyl serine; PI, propidium iodide; DTSSP, (3,3′-dithiobis [sulfosuccinimidylpropionate]); αCP, α chain connecting peptide; TM, transmembrane.

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It is clear that, unlike that induced by the CD20 mAb, MHC II mAb-induced cell death is not mediated by the complement-dependent cytotoxicity (CDC) or antibody dependent cell-mediated cytotoxicity (ADCC) [10]. Instead, it is an effect of signaling by MHC II [11]. The exact signaling mechanism by which MHC II transmits cell death signals remain controversial [7]. Both caspase-dependent and -independent MHC II-mediated cell death have been reported [12,13]. The involvement of CD95 (Fas) is also contradictory [7,14,15].

A substantial literature indicates that MHC II can transduce signals via at least two mechanisms. In resting mouse B cells, MHC II aggregation leads to activation of cAMP generation and stimulation of certain PKC isoforms via a mechanism that requires the β chain cytoplasmic tail [16]. In B cells that have been activated by IL-4, MHC class II is associated with Igα/Igβ heterodimers [17]. TCR aggregation of MHC II on these cells leads to tyrosine phosphorylation of Igα/β and activation of Lyn and Syk, and downstream effectors, leading to mobilization of calcium and activation of MAPKs [17]. It is unclear which, if either, of these signaling pathways is involved in MHC class II transduction of death signals.

Here we investigated MHC II structural requirements for mediation of death signaling. We utilized the K46 murine B lymphoma, which exhibits the biochemical signaling phenotype of activated murine and human B cells. We found that the MHC II α chain connecting peptide region encodes information for Igα/β association as well as signaling of the cell death response and calcium mobilization.

2. Materials and methods

2.1. Cell culture

Murine B lymphoma line K46 was maintained in IMDM supplemented with 5% FCS (HyClone, Logan, UT), L-glutamine (2 mM), penicillin (100 UI/ml), 100 μg/ml streptomycin, gentamycin (50 μg/ml), sodium pyruvate (1 mM) and 2-ME (50 μM). Cultures were incubated at 37 °C in humidified air containing 7% CO_2. All culture reagents were from Life Technologies (Gaithersburg, MD).

2.2. Cell death

To induce cell death, K46 cells were suspended in complete IMDM medium containing 5% FCS at a concentration of 10^6 cells/ml. Cells were then transferred into 96-well plate (round bottom) at 100 μl cells per well. Cells were cultured at 37 °C for 10 min, after which biotinylated MHC II mAbs were added into the wells. Cells were cultured again for another 12 min. Avidin was then added into the wells. Cells were cultured for indicated time until cell death was measured.

Annexin V was used to measure phosphatidylserine (PS) externalization; Annexin V—Alexa Fluor 488 (1 μl) (cat# A-13201, Molecular Probes) was directly added into 100 μl cells in the 96-well plate. Cells were cultured at 37 °C for 5 min before transfer into FACS tubes containing 150 μl FACS buffer. Cells were cooled on ice. Propidium iodide (PI) (2 μg/ml) was added into cells immediately before cell analysis using a FACSCalibur.

DiOC6 (3) was used to measure mitochondrial membrane potential. DiOC6 (3) (50 nM) was added directly in 10^5 cells in 100 μl complete IMDM medium in a 96-well plate. Cells were cultured at 37 °C for 30 min before transferred into FACS tubes as above. Cells were cooled on ice. PI (2 μg/ml) was added to the cell suspension immediately analysis on a FACSCalibur. DiOC6 (3) low staining cells were counted as dead cells.

Propidium iodide (PI) was used to measure plasma membrane integrity. Cells were transferred from 96-well plate to FACS tubes as above. PI (2 μg/ml) was added into cell suspensions immediately prior to analysis on a FACSCalibur.

2.3. Western blotting and Abs

K46 cells were lysed in 0.33% CHAPS lysis buffer (150 mM NaCl, 10 mM Tris pH 7.5, 10 mM sodium pyrophosphate, 2 mM Na_3VO_4, 10 mM NaF, 0.4 mM EDTA, 1 mM PMSF, and 1 μg/ml each of aprotinin, α_1-antitrypsin, and leupeptin) on ice for 1 h or overnight. Immunoblotting was done as before [17]. MHC II was detected using rabbit polyclonal Ab (#13110) or mouse mAb 10.2.16. CD19 was detected using rabbit polyclonal Ab (#12416). Igα and Igβ were detected using in-house rabbit polyclonal antibodies directed to the cytoplasmic tail of Igα or Igβ. Other Abs used are m5/114, d3/137, 39j, gk1.5, okt8, anti-AKT Ab (#9272, Cell Signaling Technology) and p^{Ser473}-AKT Ab (#9271, Cell Signaling Technology).

To detect cell surface MHC II-CD19 association, K46 cells (2×10^7 cells/ml in PBS) were first treated with DTSSP (3,3′-dithiobis [sulfosuccinimidylpropionate]) (400 μM) (Pierce, cat#21578) at 4 °C for 1 h. Glycine (50 mM) was then added to the cell suspension for another 5 min at 4 °C to stop the DTSSP reaction. The cells were then spun down and lysed in modified RIPA buffer (1% NP-40, 50 mM Tris–HCl pH7.4, 0.5% Na-deoxycholate, 150 mM NaCl, 0.1% SDS, 1 mM PMSF, 0.4 mM EDTA, 1.8 mg/ml iodoacetamide, 10 mM NaF, 2 mM Na_3VO_4, 1 μg each of aprotinin, leupeptin, pepstatin) at 4 °C for 1.5 h or overnight. Immunoprecipitation was done as before [17].

2.4. Analysis of intracellular free calcium concentration ($[Ca^{2+}]_i$)

For measurement of free intracellular calcium, cells were loaded with Indo-1AM (Molecular Probes, Eugene, OR), followed by washing and suspensing in IMDM supplemented with 3% FCS. The cells were analyzed (10^6 cells/ml) before and after stimulation via crosslinking with avidin (2 μg/ml). Data were analyzed by Flow-Jo software (Tree Star, Inc., San Carlos, CA).

2.5. Constructs

To construct I-A^k α mutant that lacks the cytoplasmic tail, a stop codon mutation was introduced into the first cytoplasmic amino acid in I-A^k α chain using QuickChange XL Site-directed Mutagenesis Kit from Stratagene. The two primers used were: