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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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Summary

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; Ig-α/β

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

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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].

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 Ig-α/Ig-β 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.

Materials and Methods

Cell culture

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

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 (1μl) (cat# A-13201, Molecular Probes) was directly added into 100μl cells in the 96-well plate. Cells were cultured at 37°C for 5 min before transfer into FACS tubes containing 150μl FACS buffer. Cells were cooled on ice. Propidium idodide (PI) (2μg/ml) was added into cells immediately before cells analysis using a FACSCalibur.

DiOC6 (3) was used to measure mitochondrial membrane potential. DiOC6 (3) (50nM) was added directly in 10^5 cells in 100μl 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 (2μg/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 (2μg/ml) was added into cell suspensions immediately prior to analysis on a FACSCalibur.

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 1hr 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 (2×10^7cells/ml in PBS) were first treated with DTSSP (3, 3'- Dithiobis [sulfosuccinimidylpropionate]) (400μM) (Pierce, cat#21578) at 4°C for 1h. Glycine (50mM) 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, 50mM Tris-HCl pH7.4, 0.5% Nadeoxycholate, 150mM NaCl, 0.1% SDS, 1mM PMSF, 0.4mM EDTA, 1.8mg/ml iodoacetamide, 10mM NaF, 2mM Na_3VO_4, 1μg each of aprotinin, leupeptin, pepstatin) at 4°C for 1.5h or overnight. Immunoprecipitation was done as before [17].

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).

Constructs

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

Forward: $^{5'}$TCTTCATCATTCAAGGCCTGTGATCAGGTGGCACCTCCAG$^{3'}$

Reverse: $^{5'}$CTGGAGGTGCCACCTGATCACAGGCCTTGAATGATGAAGA$^{3'}$

To construct I-A$^{\kappa}$ β mutant that lacks the cytoplasmic tail, a stop codon mutation was introduced into the first cytoplasmic amino acid in I-A$^{\kappa}$ β chain using the same QuickChange Kit. The two primers used were:

Forward: $^{5'}$CGGGCTTGGCCTTTTCATCTGACACAGGAGTCAGAAAGG$^{3'}$

Reverse: $^{5'}$CCTTTCTGACTCCTGTGTCAGATGAAAAGGCCAAGCCCG^{3}

The mutated bases are underlined.

To make the connecting peptide mutants, the α (amino acids 182–195) or β (amino acids 189–198) chain of I-A^{k} was replaced by the corresponding domain of H-2D^{d} (amino acids 302–310). The primers used were:

5' α^{k}, 5'-CCCAAGCTTAACACTGTGGTGTGTGCCCTG-3';

5' β^{k}, 5'-CCCAAGCTTAACAAGATGTTGAGCGGCATC-3';

and 3' H-2D^{d}, 5'-CCCAAGCTTGGTGGATGAAGG-3'.

The transmembrane and cytoplasmic domains of H-2D^{d} were cloned from K46 cell cDNA library using the primer pair:

5’ primer, 5’-CGGGATCCGAGGAGCCTCCTTCA-3’

3’ primer, 5’-CGGAATTCTCACACTTTACAATC-3’

The resulting fragment was digested and cloned into the EcoRI site of pBluescript SK (Stratagene). The extracellular domains of the I-A^{k} alpha chain (amino acids 1–195) or beta chain (amino acids 1–198) was fused to the cytoplasmic and transmembrane domains of H-2D^{d} (amino acids 311–365)(designated αTM and βTM, respectively). Resulting products were cloned as completed chimeras into the EcoRI site of pMXI-egfp. To facilitate cloning, the valine at position 311 and the isoleucine at position 313 of H-2D^{d} were changed conservatively to glycine and leucine, respectively.

Knock-down of Igβ expression using shRNA

The shRNA was designed using pSicoOligomaker 1.5[18], which targets a sequence (5’-GGAGTTCTCTGGGGATAGA-3’) in the 3’-UTR region of the B29 gene (201bp after the stop code). The annealed oligos were ligated into pLL3.7 through Hpa I and Xho I sites. Lentiviruses were generated as described [18].

Retrovirus generation and K46 cells infection

Retroviruses containing I-A$^{\kappa}$ α and β chains were produced using the amphotropic Phoenix packaging cell line and used to infect K46 cells as described previously [19]. Cell surface I-A$^{\kappa}$ expression was confirmed by flow cytometry using the biotinylated anti-I-A$^{\kappa}$ mAb 39J or 10.2.16 and Streptavidin-PE. Cells expressing equivalent levels of I-A$^{\kappa}$ were sorted and propagated for use in experiments.

Results

Anti-MHC II mAb induces rapid cell death in K46 murine B lymphoma cells

Monoclonal anti-MHC II antibodies reportedly induce death of cells exhibiting activated phenotypes [10]. The murine B cell line K46 was used previously to study *in vitro* MHC II signaling [20]. Cell surface staining showed these cells exhibit phenotype similar to activated B cells with high surface expression of CD80 and CD40 (data not shown). Similarly, in these cells, MHC II is associated with Igα/β and aggregation of MHC II leads to tyrosine kinase

activation and calcium mobilization[17]. We determined whether MHC II mAb can induce death of these cells.

K46 cells were incubated with biotinylated anti-mouse I-$A^{b, d, q}$/$E^{d, k}$ specific mAb M5/114 for 12 min at 37°C in culture, cells were washed and bound antibodies were cross-linked using avidin. Cell death was determined by Annexin V, DiOC6 (3) and PI staining. After 1.5h, K46 cells had significantly increased PS externalization, decreased mitochrondrial potential and loss of plasma membrane integrity (Fig 1a). Based on Annexin V^+ or DiOC6 $(3)^{low}$ staining, the cell death reached 40% of the total cells after 1.5h (Fig 1a). The time-course analysis showed that MHC II mediated cell death in K46 is fast with peak cell death observed by Annexin V and DiOC6(3) staining at about 1.5h (Fig 1b, left panel). The death response is also dose dependent, reaching maximum at 20μg/ml biotin-M5/114 (Fig 1b, right panel).

Previous studies in human B cells suggested that MHC II mAb-induced cell death is not dependent on IgG receptor (FcR) signaling [21]. K46 cells express very low levels of surface FcR (data not shown). Directly cross-linking of cell surface FcR did not induce cell death (Fig 1c). Pre-incubating cells with anti- FcR Ab 2.4G2 did not block M5/114 induced cell death (Fig 1c). Thus MHC II mAb induced cell death in K46 cells is not dependent on FcR.

Truncating the cytoplasmic tail of either α or β chain does not affect MHC II-mediated cell death

Though it is generally accepted that anti-MHC II mAbs induce cell death via signaling [11], the exact signaling events involved in MHC II cell death remain controversial. K46 cells do not express surface CD95 (data not shown) yet there have been reports of CD95 (Fas) involvement in MHC II-mediated cell death [15]. Other reports have described roles of caspase-dependent [22] and caspase-independent pathways[13]. PKC activation is reportedly for death responses of human B cells [23] but not primary human plasmacytoid DC[24]. Most of these conclusions came from studies using specific chemical PKC inhibitors. To begin dissection of upstream pathways involved in MHC II-mediated death, we conducted a mutational analysis of MHC class II.

The cytoplasmic tails of MHC II have been shown to be linked to PKC activation and cAMP production [16]. We generated K46 cells expressing I-A^k mutants that lack the entire cytoplasmic tail of the α (α-cy/β-wt) or β chain (α-wt/β-cy) (Fig 2a). K46 cells express endogenous I-A^d and I-E^d. I-A^k and I-A^d chains do not mis-pair in K46 cells as indicated by mAb staining (data not shown) [25]. The α-cy/β-wt or α-wt/β-cy was introduced into K46 cells. Cell surface I-A^k expression was confirmed by 39J staining and matched to wild type I-A^k by sorting (data not shown). Western blotting using anti-I-A^k β chain specific mAb 10.2.16 showed that β-cy is smaller than wild type β chain (Fig 2b). Cell death was induced by 39J mAb and determined by PI staining. No significant death response defect was seen in either I-A^k mutant (Fig 2c). We generated cells expressing I-A^k mutant that has truncation on both cytoplasmic tails (α-cy/β-cy). No significant death defect was seen upon 39J stimulation (Fig 2d). Similar results were observed when using 10.2.16 mAb (data not shown). We thus concluded that the cytoplasmic sequence of I-A^k is not required for MHC II-mediated cell death in K46 cells.

Impaired cell death response in K46 cells expressing I-A^k α chain connecting peptide (I-A^k αCP) mutant

To further define structural requirements for MHC II mediated cell death, we replaced the transmembrane domain (TM) and the connecting peptide region of either I-A^k α or β chain with the corresponding region in MHC I (Fig 3a). We examined the death response induced by 39J mAb in these cells. Replacing the TM of either α or β chain did not affect 39J-induced

cell death (Fig 3b, left panel). Replacing the β chain connecting peptide region also did not affect the death response (Fig 3b, right panel). However, replacing the I-A^k αCP region resulted in 50% decrease in 39J induced cell death (Fig 3b, right panel). Similar results were observed using biotinylated 10.2.16 mAb (data not shown). We conclude that I-A^k αCP region contributes to the death response.

The I-A^k α chain connecting peptide region is required for the MHC II-mediated calcium response and Igα/β association

Towards an understanding of how the I-A^k αCP contributes to the death signal, we investigated disruption of primary signaling events in the I-A^k α*CP* mutant. We found that the I-A^k αCP mutant did not flux calcium through the mutant I-A^k (Fig 4a). Other I-A^k mutants, αTM, βTM, βCP, had normal calcium response through I-A^k (data not shown).

We hypothesized that the I-A^k α*CP* region affects cell death and calcium response through associated cell surface transducer(s). MHC II has been reported to associate with many surface proteins including Igα/β in mouse B cells[17], and CD19, CD20, CD40 in human B cells[26, 27]. We found that the I-A^k αCP mutant no longer associated with Igα, and this effect was partially replicated in the α^k4A in which all four glutamic acid residues in the α*CP* region were replaced with alanines. β-CP mutants retained Igα/β association (Fig 4b).

MHC II signaling is also dependent upon CD19 [20]. Using anti-I-A^k β chain mAb 10.2.16 and anti-I-A/$E^{b/d}$ mAb m5/114, we confirmed that CD19 is associated with both I-A^k and I-A^d/E^d in these cells (Fig 4c). To determine that they are associated on the cell surface, we developed a method that combined chemical crosslinker DTSSP and modified RIPA lysis buffer. Cells were first treated with DTSSP, a membrane impermeable chemical crosslinker, to achieve surface crosslinking. The DTSSP reaction was stopped after 1h by the addition of glycine and the cells were lysed in Tris buffer based RIPA buffer that contains 0.1% SDS. RIPA containing SDS disrupts most protein-protein interaction during cell lysis. Thus using this method, any protein association with MHC II is due to DTSSP cross-linking.

Using this method, we found CD19 and Igα/β co-immunoprecipitated with MHC II in a DTSSP dependent manner (Fig 4d), which suggested both interact with MHC II on cell surface. We next investigated CD19/MHC II association in the I-A^k α*CP* mutant. The mutation in the α chain connecting peptide region did not affect DTSSP dependent CD19/MHC II association (Fig 4e). Thus, MHC II/CD19 association does not require the I-A^k α*CP* region.

MHC II mediated calcium mobilization requires Ig α/β

The I-A^k α*CP* region is required for the cell death, calcium response and Igα/β association suggesting linkage among these responses. To further investigate this linkage we generated an Igβ knockdown K46 cell line. Surface IgM expression was down more than 95% in Igβ knockdown cells while the MHC II expression was unchanged (Fig 5a). Moreover, there was no detectable DTSSP dependent Igβ association with MHC II in the knockdown cells while the CD19 expression and association was not altered (Fig 5b). The association of Igα with MHC II also decreased dramatically though the expression of Igα only slightly decreased (Fig 5b). Thus, knock-down of Igβ eliminates surface MHC II-Igα/β association consistent with the effect of I-A^k αCP mutation.

We next determined whether MHC II-mediated calcium responses occur in the Igβ knockdown cells. K46 cells express NP-specific surface IgM. Cells were activated by anti-I-A^d mAb biotin-D3.137 and avidin or NP antigen. The K46 cells expressing a control shRNA targeting the luciferase gene responded to NP antigen and D3.137 mAb activation well (Fig 5c). But the Igβ knockdown cells were unable to mobilize calcium upon either NP antigen (due to its loss

of surface IgM) or MHC II crosslinking (Fig 5c). We concluded that MHC II association with Igα/β is essential for MHC II-mediated calcium response, and the lack of calcium response in the I-A^k αCP mutant is due, to its loss of interaction with Igα/β.

MHC II association with Igα/β is not required for the death response

We assessed whether the impaired death response in the I-A^k αCP mutant is due to its loss of interaction with Igα/β. Cell death was induced in Igβ knockdown K46 cells as well as K46 cells expressing control shRNA with biotin-D3.137 and avidin and measured by PI staining. To our surprise, the Igβ knockdown and wild type cells underwent a similar death response (Fig 6a). This indicated that the impaired death response observed in the I-A^k αCP mutant is not due to its loss of interaction with Igα/β.

AKT activation is another prominent signaling event activated by MHC II aggregation [12]. Crosslinking MHC II in K46 cells activates CD19-PI3K pathway [20], which likely leads to AKT activation [28]. We were interested whether AKT activation is altered by Igβ knockdown. Cells were activated with biotin-D3.137 and avidin, and whole cell lysates were probed with phosphor-ser^{473} AKT Ab. As shown in Fig 6b, Igβ knockdown cells have normal AKT activation. Therefore MHC II associated Igα/β is not required for AKT activation, consistent with the observation that MHC II/CD19 association is unchanged in Igβ knockdown cells (Fig 5c).

Discussion

Increasing data supported the biological significances of MHC II signaling as well as its potential as a therapeutic target [4]. Multiple signaling pathways are activated by anti-MHC II mAbs crosslinking including cAMP production, PKC activation, calcium mobilization, tyrosine phosphorylation and AKT activation [4]. However, it is not clear which of these signaling pathways are important for the therapeutic effect of anti-MHC II mAbs. To start address this question, we undertook analysis of class II structural requirements for transduction the death signals. We determined that it is the MHC class II α chain connecting peptide region, not the MHC class II TM and cytoplasmic domains, is required for death signaling.

MHC II contains a short cytoplasmic tail that is important for many signaling events including MHC II mAb induced PKC activation and cAMP production [16]. We showed here that the cytoplasmic tails and the TM regions of MHC II are not required for the cell death response (Fig 2, 3). Thus, our results indicate that the signaling events initiated by these regions are not required for the death response. They also suggested that the death signaling must be transduced by MHC II associated cell surface molecule(s).

The MHC II-mediated death response, as well as the calcium response, is significantly impaired in the αCP mutant (Fig3&4). We hypothesized that the transducer(s) that mediates death and calcium signaling maybe associated with MHC II through this region. We found in this report that the Igα/β association is through the αCP region (Fig 4). Furthermore, knock-down Igβ expression by shRNA eliminated MHC II mediated calcium response (Fig 5). These data confirmed early report that MHC II mediated calcium signal is dependent on its association with Igα/β[17] and further showed that this signal is via the I-A^k αCP region. It is noteworthy that MHC II-Igα/β association is different from the IgM-Igα/β association which is mainly mediated by IgM transmembrane region[29].

Interestingly, the Igβ knock-down cells had normal cell death responses (Fig 6). This suggested the impaired death signaling in αCP mutant is not due to its loss association with Igα/β. Some other MHC II associated transducer(s) must be responsible for the defect. Multiple cell surface proteins are reportedly associated with MHC II including Igα/β, CD19, CD20, CD40, and CD9-

CD37-CD81 tetraspanin proteins [17,26,27,30,31]. The Igβ knock-down cells have normal CD19 association (Fig 5). However, CD19, CD20 and Igα/β are B cell specific proteins and since MHC II-mediated cell death is also observed in DC[14], these B cell specific proteins can not be required for the death signaling. Tetraspanins have very short cytoplasmic tails and no defined signaling motifs. CD40 is found on both B cells and DC. However, CD40 actually protects cells from MHC II-mediated cell death [14]. Thus it seems likely that other, as yet undefined, associated proteins must be responsible for transmitting the death signals. We are currently exploring this possibility and have defined a previously unknown MHC II associated tetraspan protein that contains multiple signaling motifs in its cytoplasmic tail and is required for the death response (Jin, L and Cambier, JC, manuscript in preparation).

It is interesting to notice that MHC II transduced death signaling does not require calcium mobilization, tyrosine phosphorylation (data not shown) [32] and all other signaling events that require the cytoplasmic tails of MHC II. On the other hand, loss of Igβ expression eliminated calcium response (Fig 5) and tyrosine phosphorylation (data not shown) but does not affect cell death and AKT activation (Fig 6). These observations suggested that MHC II engages multiple parallel signaling pathways that may be initiated by different MHC II associated membrane proteins (Fig 7). Calcium signaling is transmitted by MHC II associated Igα/β. AKT is activated by MHC II-associated CD19, and the death response is initiated by some unknown MHC II-associated protein (Fig 7).

The implications of the accessory molecule coupling to parallel MHC II signaling pathways model are twofold. First, most of the well-known MHC II-associated signaling proteins, including CD19, CD20, Igα/β are B cell specific. This suggests that the response to MHC II crosslinking in B cells and DCs might be different. In fact, though MHC II engagement can kill both B cell lymphoma and DCs *in vitro*[5], MHC II-mediated antigen presentation to $CD4^+$ T cells by DCs and B cells have different outcomes *in vivo*. Mature DCs die after antigen presentation, while B cells may be activated to participate in immune response.

Second, the findings reported here have implications for the therapeutic use of anti-MHC II mAb. Most death-inducing MHC II mAbs also activate the AKT survival pathway [12,33](Fig 7), which may decrease the efficiency of tumor killing. AKT activation is likely a result of MHC II-associated CD19-PI3K pathway [20]. An anti-MHC II mAb, designed to target the CD19 binding sites on MHC II could disrupt surface MHC II/CD19 association and initiate the death signals without the activation of AKT survival pathway. Such an agent might be a more efficient killer of tumor cells.

Future studies should be focused on identification of additional MHC II associated surface signaling proteins, particularly the surface co-receptor that is responsible for transmitting MHC II-mediated death signal.

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Abbreviations

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

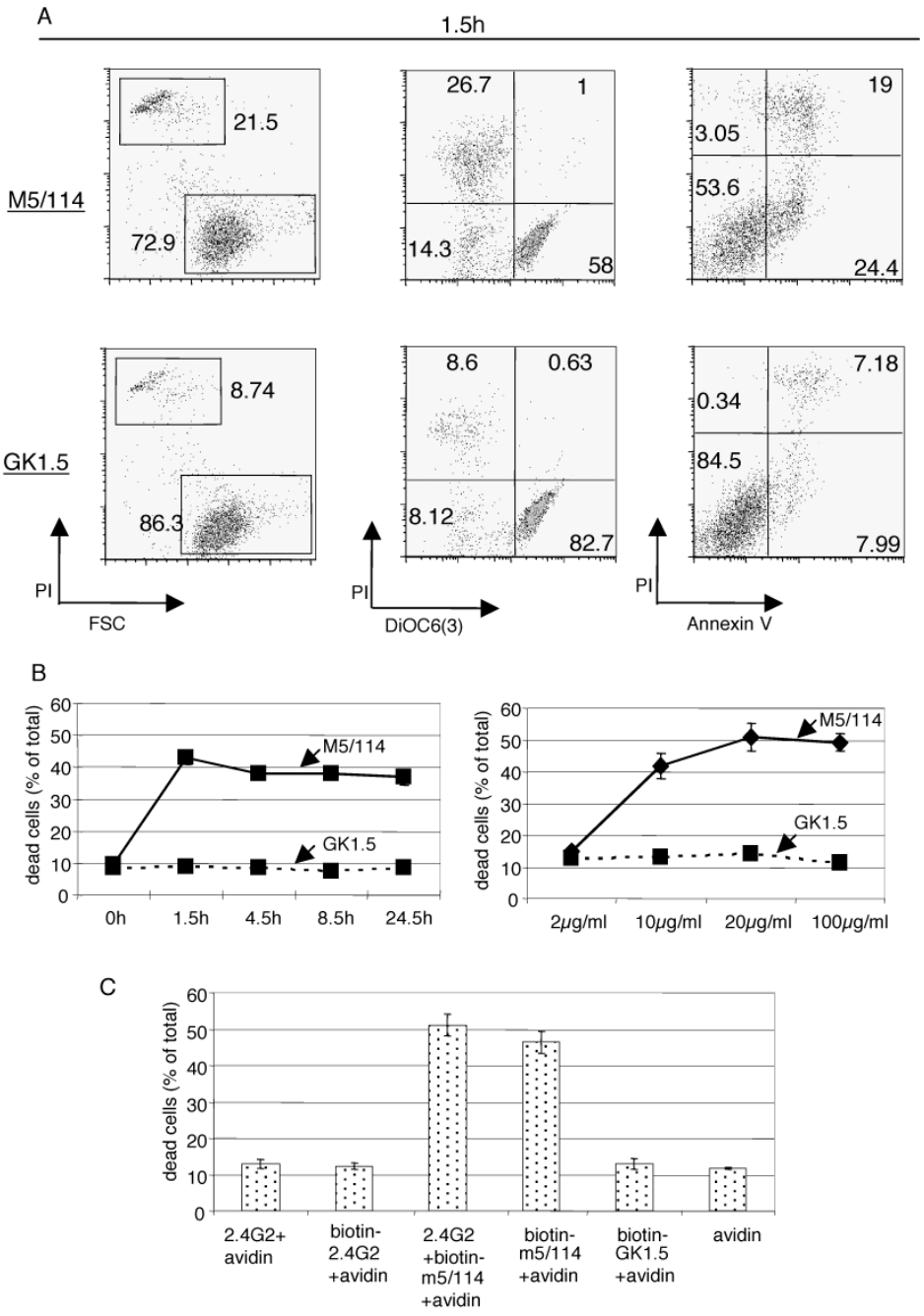

Figure 1.

MHC class II mAb induces potent and rapid cell death in K46 B lymphoma cells. A. K46 cells were treated with 10μg/ml biotinylated M5/114 (rat IgG_{2b}) mAb or its isotype control biotinylated GK1.5 anti mouse CD4 mAb (rat IgG_{2b}) followed by 20μg/ml avidin for 1.5h. Cell death was measured by PI (left two panels), PI plus DiOC6 (middle two panels) and PI plus Annexin V (right two panels) staining (n>3); B. Cells were treated as (A) at indicated doses or time. Cell death was measured by Annexin V staining (n>3); C. Cells were treated with 10μg/ml indicated biotinylated mAb and avidin for 1h or incubated first with 10μg/ml anti-FcR mAb 2.4G2 (rat IgG_{2b}) for 15 min in culture followed by 10μg/ml indicated

biotinylated mAb and avidin for 1h. Cell death was measured by Annexin V staining (n>3). Error bars represent SD of triplicates.

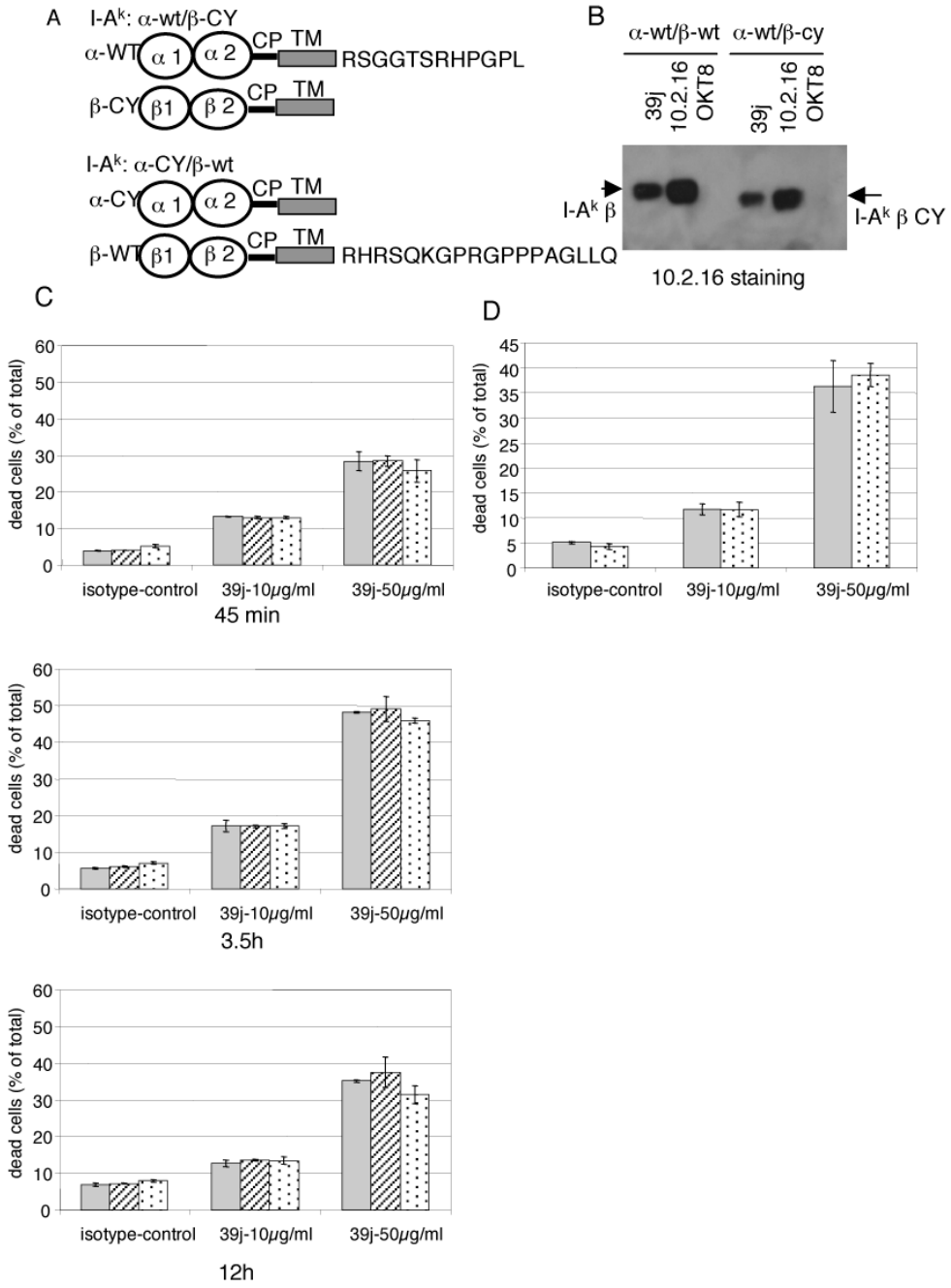

Figure 2.

The cytoplasmic tails of MHC II are not required for the death response. A. I-A^k mutants that lack the cytoplasmic tails of I-A^k α or β chain; B. I-A^k molecules were immunoprecipitated from indicated cells with I-A^k specific mAbs 10.2.16 or 39J. The blot was stained with 10.2.16 mAb (n=3); C. Cell death in K46 cells expressing wt, α(cy)/β(wt) and α (wt)/β(cy) I-A^k were induced by 10μg/ml or 50μg/ml biotinylated 39J (mouse IgG_{2a}, anti-mouse I-A^k α chain specific mAb) for indicated time. Cell death was measured by PI staining. OKT8 (mouse IgG_{2a}, anti human CD8 mAb) was used as isotype control (n>3); D. Cell death in K46 cells expressing wt or α(cy)/β(cy) was induced and measured as (C) (n>3); Error bars represent SD of triplicates.

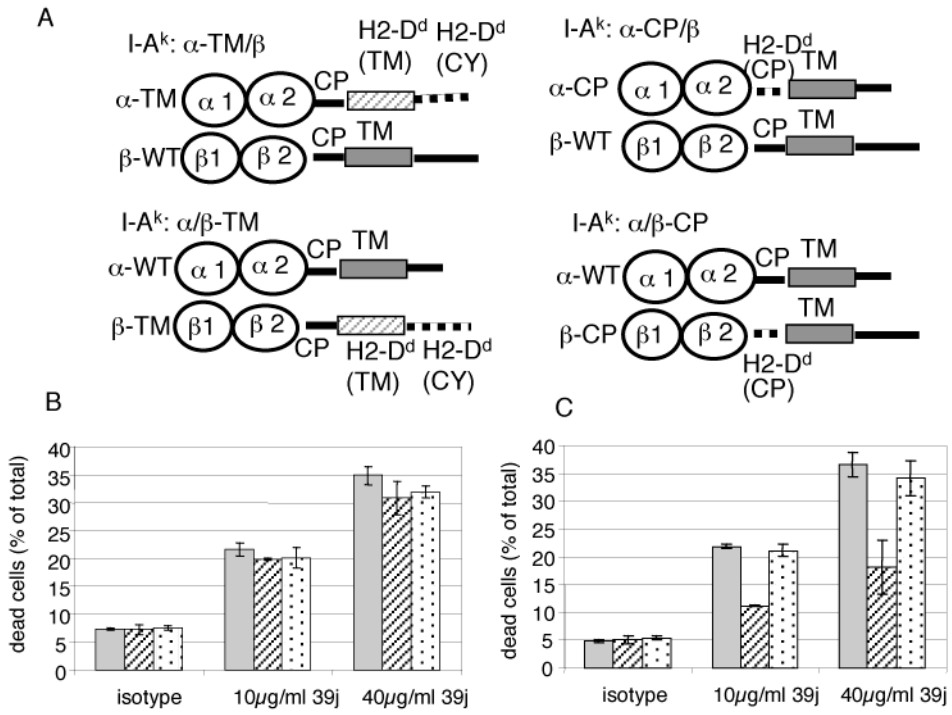

Figure 3.

The connecting peptide region of I-A^k α chain is required for MHC II mediated cell death in K46 cells. A. I-A^k mutants that had the transmembrane domain or connecting peptide region replaced by the corresponding H2-D^d region; B. Cell death was induced in K46 cells expressing wild type (▩), α(TM) (□) or β(TM) (□) mutant I-A^k by biotinylated 39J plus avidin for 4h. Cell death was measured by PI staining. OKT8 was used as an isotype control (n>3); C. Cell death was induced as B in cells expressing wild type (▩), α(CP) (□) or β (CP)(□) mutant I-A^k (n>3); Error bars represent SD of triplicates.

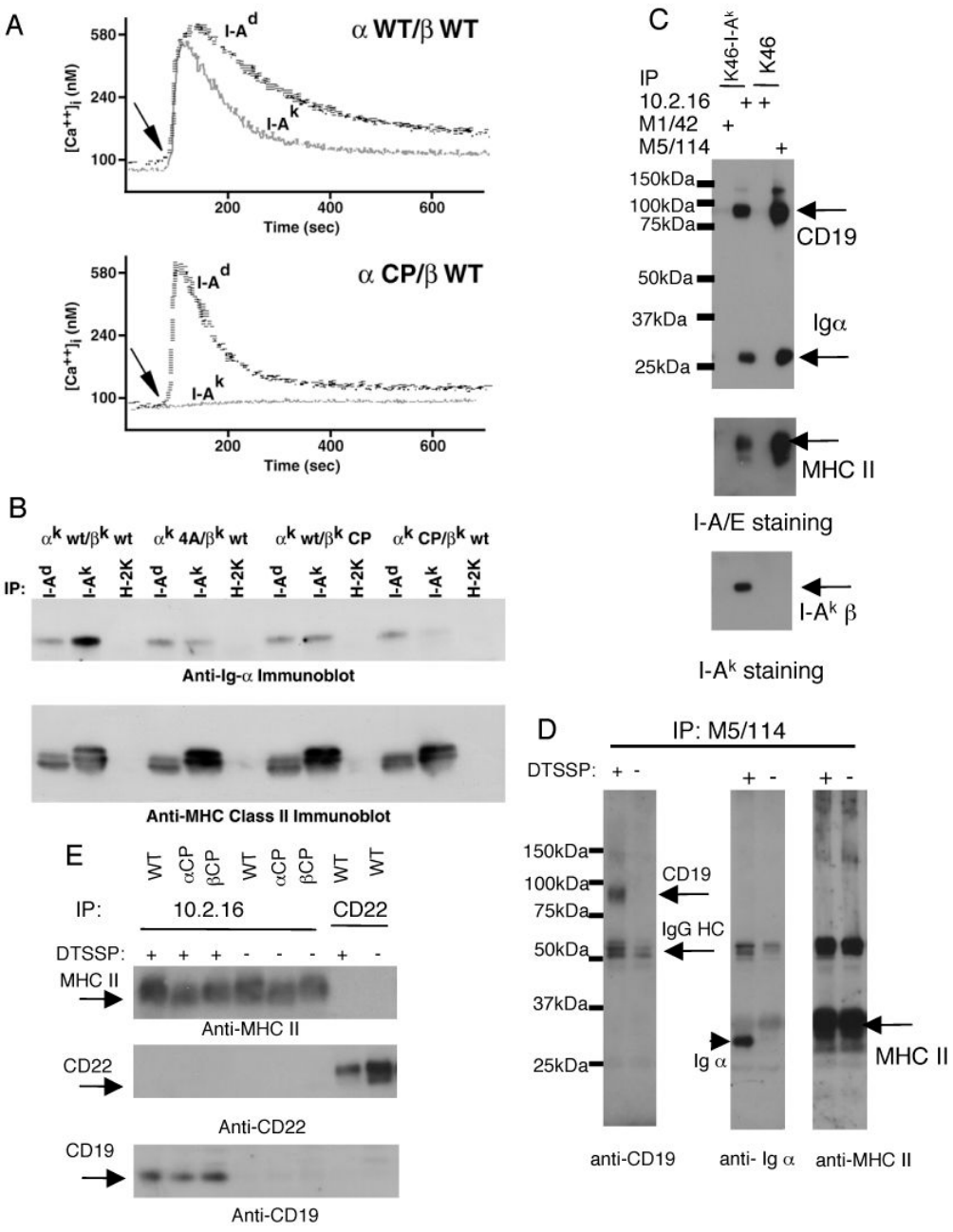

Figure 4.

The α chain connecting peptide region of I-A^k is required for calcium response and MHCII/Igα/β association. A. K46 cells expressing indicated I-A^k mutants were activated with biotinylated anti-I-A^d (endogenous MHC class II) mAb d3.137 or anti I-A^k mAb 39J (n>3); B. I-A^d, I-A^k, and H2-K were immunoprecipitated from indicated K46 cells. The blots were probed with Abs against Ig-α or MHC II (n>3); C. I-A^d, I-A^k, and H2-K were pull down with indicated mAb from parental K46 or K46 cells expressing I-A^k. The blot was probed with Abs against CD19, Igα, I-A^d and I-A^k (n=4); D. K46 cells were treated with DTSSP or not, then lysed in modified RIPA buffer. MHC II was pull down by M5/114 mAb and the blot was probed with indicated Abs (n>3); E. The indicated cells were treated with DTSSP as (D). I-

A^k and CD22 were pull down with Abs. The blots were probed with Abs against MHC II, CD19 and CD22 (n=3).

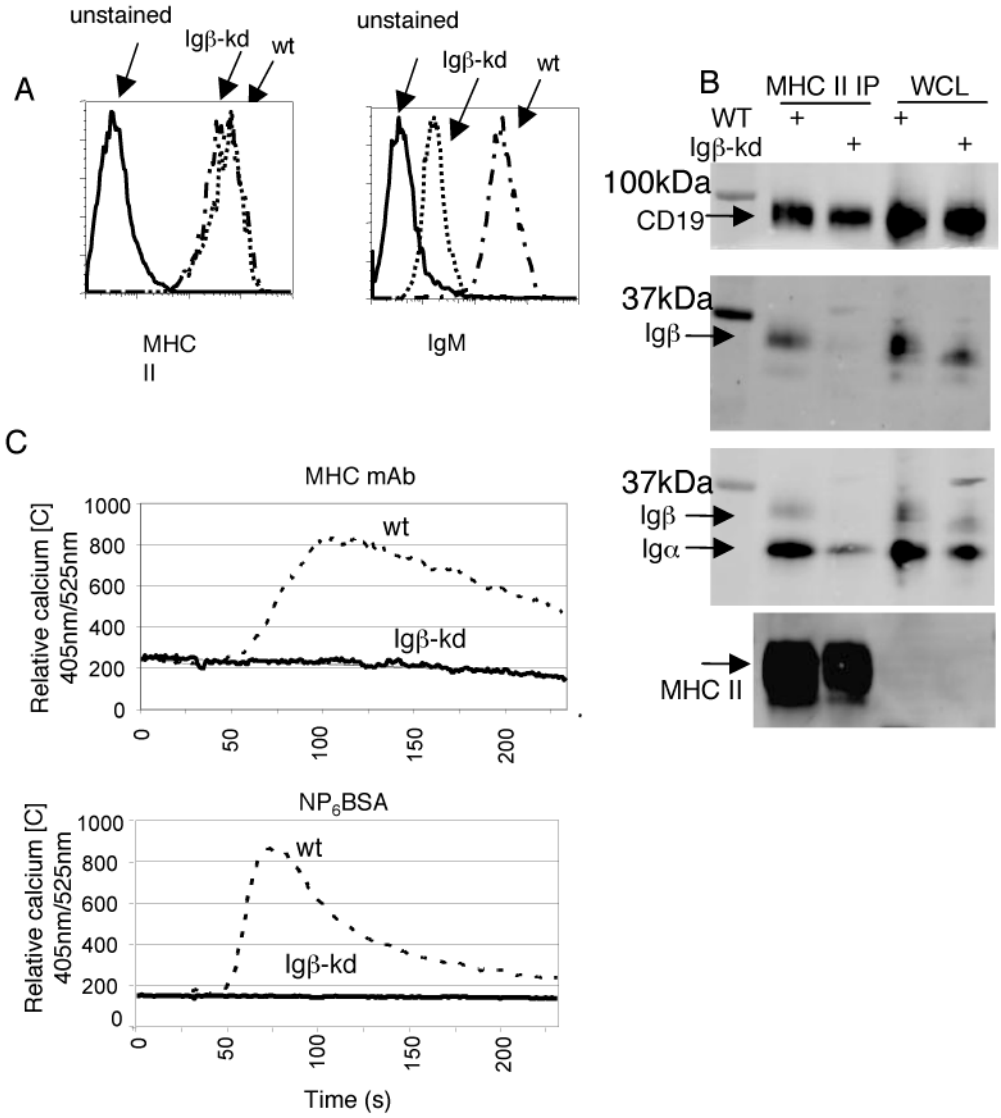

Figure 5.

Igβ is required for MHC II mAb induced calcium response. A. K46 cells expressing control shRNA targeting the luciferase gene (WT) or Igβ-kd K46 cells were stained for surface expression of MHC II and IgM (n>3); B. WT or Igβ-kd cells were treated with DTSSP as (fig 4d). MHC II was pull down with M5/114 mAb. The blot was probed with indicated Abs (n=4); C. WT and Igβ-kd cells were loaded with Indo-1 AM. Cells were activated with NP_6BSA (100ng/μl) or biotinylated D3.137 (20μg/ml) and avidin (20μg/ml). (n=3).

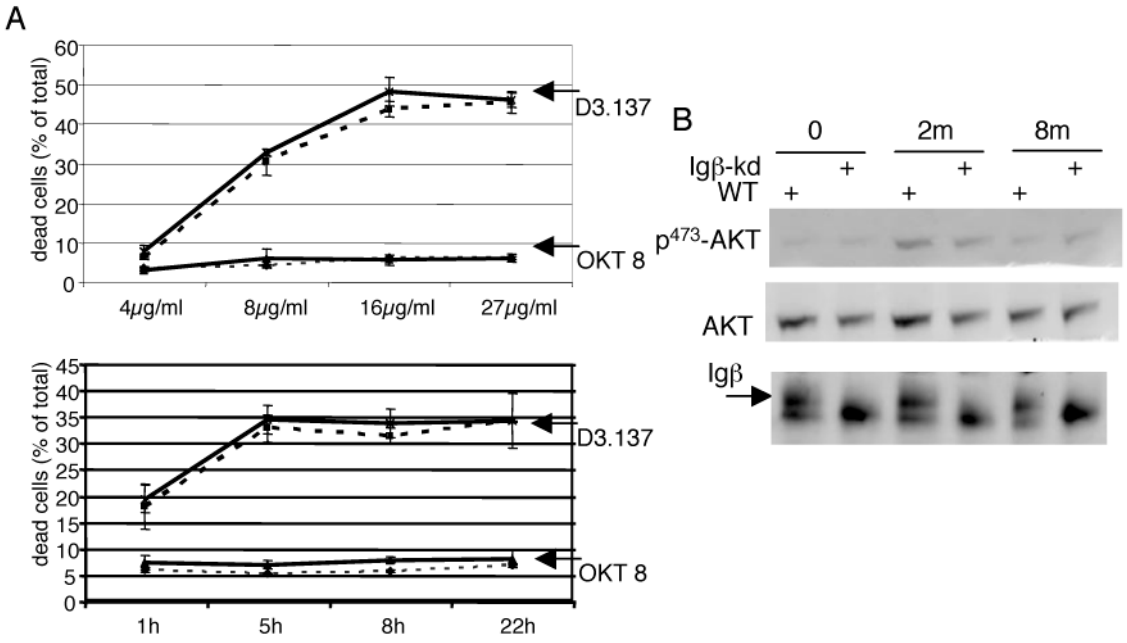

Figure 6.

Igβ is not required for MHC II mediated cell death and AKT activation. A. WT (expressing control shRNA) (dot line) or Igβ-kd (expressing shRNA targeting Igβ) (solid line) cells were stimulated with indicated doses of biotinylated D3.137 (mouse IgG_{2a}) or isotype control OKT 8 (mouse IgG_{2a}) and avidin for indicated time. Error bars represent SD of triplicates (n=3); B. WT and Igβ-kd cells were activated with biotin-D3.137 (20μg/ml) and avidin (20μg/ml) for indicated time. The whole cell lysate were probed with indicated Abs (n=3);

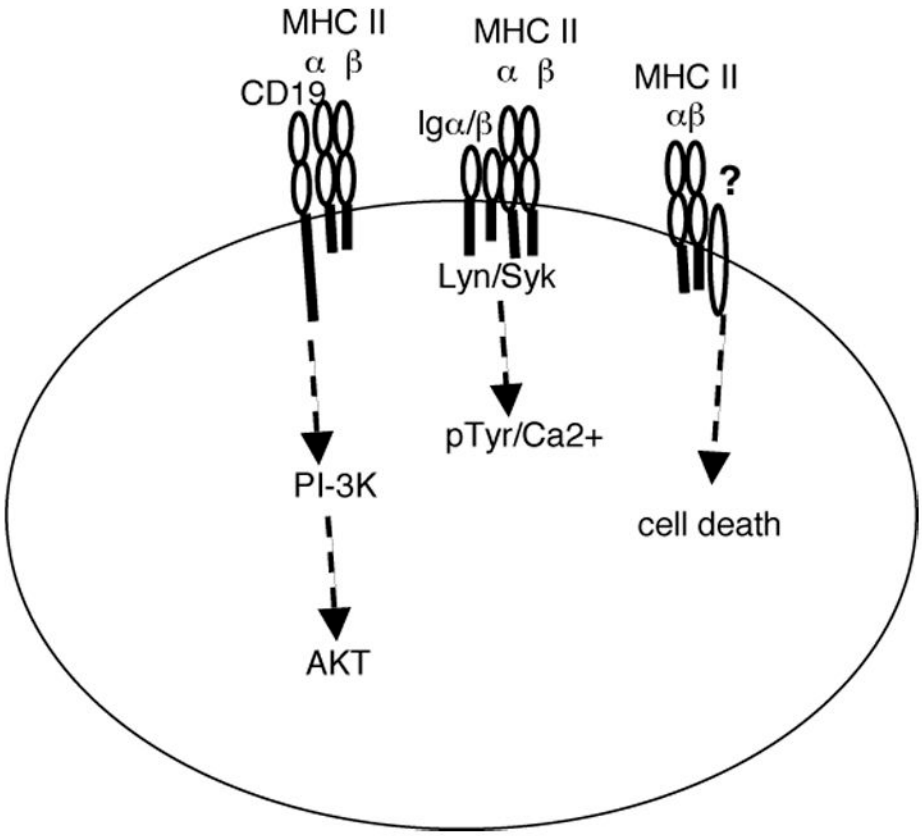

Figure 7.
Model: Parallel signaling induced by different MHC II associated accessory molecules.