Activation of a T cell hybridoma by an alloligand results in differential effects on IL-2 secretion and activation-induced cell death

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The molecular nature of the interaction of T cell receptors (TCR) with alloligands is not well understood. Although a role for groove-bound peptide(s) has been clearly demonstrated for major histocompatibility complex (MHC) class I alloreactivity, this has not been established for MHC class II-induced alloresponses. In the present study, we have analyzed the interaction of a nominal peptide-self MHC complex and of an alloligand with their cognate TCR (1934.4 TCR for autoantigen recognition and qCII85.33 TCR for allorecognition). Our results demonstrate that 1934.4 TCR recognition of the N-terminal epitope of myelin basic protein (Ac1-11, Ac=acetylated at position 1) complexed with the MHC class II molecule I-A^u involves contacts with both chains of the MHC molecule. In contrast, qCII85.33 TCR recognition of an allopeptide:I-A^u complex appears to predominantly involve the β chain of the MHC molecule. Thus, the two TCR appear to have different footprints on the I-A^u molecules. Unexpectedly, this differential involvement of the two chains of the I-A^u molecule affects activation induced cell death, with allostimulation resulting in poor induction of FasL expression and relatively low levels of apoptosis. Significantly, stimulation of cognate T cells with alloantigen or autoantigen results in similar levels of IL-2 secretion. The reduced apoptosis of T cells in response to allostimulation may be one of the mechanisms that favors the expansion of a relatively large repertoire of alloreactive T cells.

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1 Introduction

During their development, thymocytes undergo processes of positive and negative selection which result in the generation of a repertoire of peripheral T cells able to recognize foreign peptides in the context of self MHC molecules. This developmental program also produces a large pool of alloreactive T cells (1–10% peripheral T cells) which can recognize foreign MHC molecules [1–5]. Although much is now known about the molecular details as to how foreign peptides complexed with self MHC molecules interact with cognate T cells [6–10], the interaction of TCR with alloligands remains poorly understood. For example, the role of MHC-bound peptide in alloreactivity is still not well established and both peptide-dependent and peptide-independent stimulation have been reported [11–20]. In general, however, it is believed that the majority of alloresponses are peptide dependent. Unlike MHC class I alloreactivity much less is known about MHC class II alloreactivity which plays an equally important role in tissue rejection during transplantation.

The X-ray crystallographic structure of an alloreactive TCR complexed with its alloligand has recently been reported [21]. The 3-dimensional structure shows a diagonal orientation for the TCR-alloligand interaction analogous to that reported for TCR-nominal peptide-self MHC complexes [6–10] or for a modelled 2C-L^d/QL9 complex [22]. Thus, allo- and foreign peptide-MHC recognition by TCR appear to be similar in terms of the overall configuration of the tripartite complexes. A distinguishing feature, however, is that in the allo-complex [21] the MHC-bound peptide does not interact with the TCR α chain. Instead, the peptide shows exclusive contacts with the TCR β chain whereas in other TCR-pMHC interactions α chain contacts frequently dominate the interaction [7–10]. However, whether this "β-chain dominance" is a characteristic feature of allorecognition is as yet unknown.

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The present study describes an analysis of the interaction of two TCR with MHC class II I-A^u complexed either with the N-terminal peptide Ac1-11 derived from myelin basic protein (MBP) or with an allopeptide. Antibody inhibition analyses indicate that these two TCRs dock with different footprints onto their respective ligands, with differential involvement of the α chain of the I-A^u molecule in the interactions. Our observations suggest that the footprint of the TCR on cognate ligand can affect the extent of activation induced cell death without affecting IL-2 secretion. This may have implications for the generation of the alloreactive T cell repertoire.

2 Results

2.1 Analysis of the effects of allostimulation on qCII85.33 cells

The qCII85.33 T cell hybridoma was derived from T cells isolated from DBA/1 mice immunized with type II collagen (CII) and recognizes a CII peptide encompassing residues 260–270 in association with I-A^q [23]. Alloactivation of these cells *in vitro* with an I-A^u-expressing B cell lymphoma line PL-8 resulted in secretion of IL-2 in a dose-dependent fashion (Fig. 1). Co-incubation of qCII85.33 cells with the I-A^u-negative fusion partner M12.C3 (PL-8 is a hybridoma between M12.C3 and LPS-activated spleen cells from PL/J mice) transfected with MHC class II I-A^q did not induce secretion of detectable levels of IL-2 in the absence of exogenously added CII peptide (not shown). This demonstrates that the PL-8 induced stimulation of qCII85.33 cells is allospecific.

qCII85.33 cells readily undergo apoptotic cell death when activated with the cognate ligand (baculovirus expressed recombinant I-A^q with covalently bound bovine CII peptide; [24]) or with an antibody against the β chain of the TCR (Fig. 2 A). However, when death of these cells following stimulation with PL-8 cells was analyzed, there was no significant apoptosis despite the production of high levels of IL-2 (Figs. 1 and 2B). In contrast, when PL-8 cells presented the N-terminal peptide Ac1-11 derived from MBP to an I-A^u-restricted T cell hybridoma 1934.4, significant levels of apoptosis occurred (Fig. 2 B). AICD and IL-2 secretion by 1934.4 cells was enhanced when position 4 analogs of Ac1-11 with higher affinity for I-A^u were used (Ac1-11[4A] and Ac1-11[4Y]; Fig. 2 B and C), and this is consistent with the data of others [25]. 1934.4 and qCII85.33 cells expressed similar levels of TCR and CD4 (data not shown).

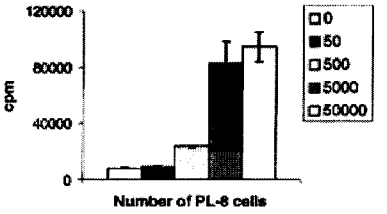
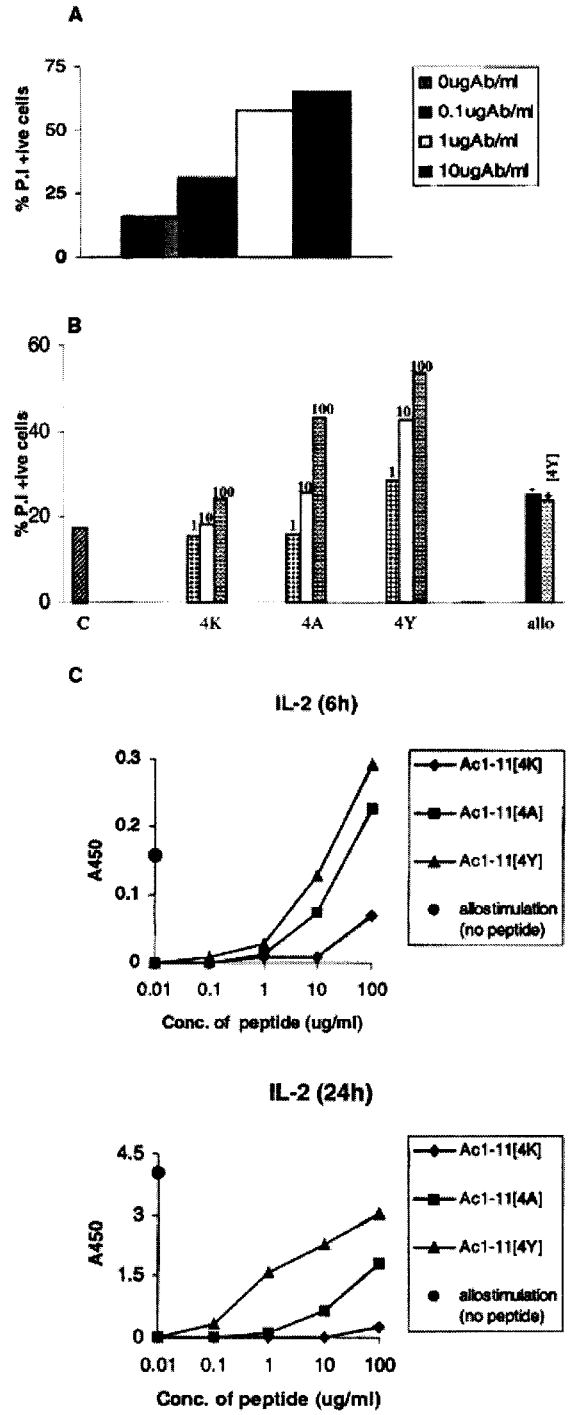


Fig. 1. Alloactivation of I-A^q-restricted CII-specific T cell hybridoma qCII85.33 by I-A^u-expressing B cell lymphoma, PL-8. 5×10^4 qCII85.33 cells were incubated with various numbers of PL-8 cells at 37 °C. After 20–24 h, culture supernatants were analyzed for IL-2 levels by measuring [^{3}H] thymidine incorporation into the IL-2-dependent cell line, CTLL-2. Data are representative of three experiments.

2.2 The footprints of the TCR-ligand interactions

To investigate the differences seen in the apoptotic responses of 1934.4 and qCII85.33 cells, we analyzed the nature of the interaction of the corresponding TCR with their respective ligands. T cell stimulation assays were carried out in the presence of antibodies specific for I-A. As can be seen from Fig. 3, a monoclonal antibody directed against the β chain of I-A, 10.2.16, efficiently blocked allostimulation of qCII85.33 cells (Fig. 3 A) and peptide-specific activation of 1934.4 cells (Fig. 3 B). In contrast, the monoclonal antibody Y3P which recognises an α chain-dependent determinant on I-A [26] inhibited peptide-I-A^u induced IL-2 secretion by 1934.4 cells but had no significant effect on alloligand-induced IL-2 secretion by qCII85.33 cells (Fig. 3 A and B). The specificity of these effects was demonstrated by using an isotype-matched control antibody (14.4.4S) which recognizes I-E (Fig. 3 B). This suggests that the qCII85.33 TCR predominantly contacts the β chain of I-A^u, whereas the 1934.4 TCR interacts with both chains of I-A^u. Alternatively, the two TCR may contact the I-A^u α chain at different sites.

The mechanism responsible for the poor induction of apoptosis in qCII85.33 cells was investigated by analyzing expression of Fas and FasL in qCII85.33 and 1934.4 cells following stimulation with their respective ligands. As can be seen in Fig. 4, activation of 1934.4 cells with Ac1-11[4Y] presented by PL-8 or Utm6.15 cells resulted in the induction of FasL expression. In contrast, qCII85.33 cells showed greatly reduced, if insignificant, FasL expression following stimulation with PL-8 cells for 5 h (Fig. 4 A). There was no significant difference in the expression of Fas by the two hybridomas (data not shown). Although low, the expression of FasL on 1934.4 cells following Ac1-11[4Y]/PL-8-induced stimulation (Fig. 4 A) was highly reproducible. Activation with the



◀ *Fig. 2.* A, AICD in qCII85.33 cells following stimulation with anti-TCR β chain antibody F23.1. Cells were activated with different concentrations of plate-bound anti-Vβ8 antibody, F23.1, resuspended in PI-containing buffer and PI-positive, *i.e.* apoptotic cells quantitated by flow cytometry. B, apoptosis in 1934.4 and qCII85.33 cells following engagement of the TCR with respective cognate ligands. 1934.4 cells were stimulated with various concentrations (μg/ml, shown above histograms) of wild type Ac1-11 (4K) and two position 4 analogs (4A, 4Y) in the presence of PL-8 cells. Control represents PL-8 and 1934.4 cells incubated in the absence of exogenous peptide. "Allo" represents allostimulation of qCII85.33 cells by PL-8 cells in the absence (–) or presence (+) of 100 μg/ml Acl-11[4Y]. AICD was determined as described in A. C, IL-2 levels following peptide specific stimulation of 1934.4 cells or allostimulation of qCII85.33 cells. T cell hybridomas were activated as described in B. After 6 h and 20–24 h *in vitro* stimulation, IL-2 levels were determined by a sandwich ELISA [44]. Data are representative of at least three experiments.

anti-TCR β chain antibody F23.1 (plate bound) led to the induction of FasL expression on both 1934.4 and qCII85.33 cells (Fig. 4 B). Despite the high expression of FasL on 1934.4 cells when using Utm6.15 cells as APC, activation-induced cell death (AICD) was higher when peptide was presented by PL-8 cells. This was due to very high expression of Fas on PL-8 cells (Fig. 4 C) which led to death of both PL-8 and T cells during *in vitro* stimulation (data not shown). In contrast, Utm6.15 cells express relatively low levels of Fas (Fig. 4 C). In addition, the possibililty that soluble mediators such as TNF-α are involved in the induction of apoptosis could be excluded by the observation that supernatants derived from

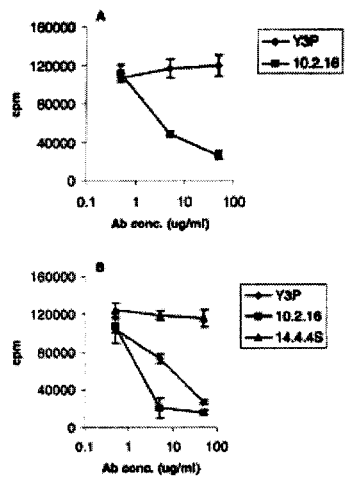


Fig. 3. Effect of anti-I-A monoclonal antibodies on IL-2 secretion by the T cells. T cell hybridomas were stimulated *in vitro* with cognate ligands in the presence of various concentrations of anti-I-A monoclonal antibodies. IL-2 levels were determined as described in Fig. 1. A, allostimulation of qCII85.33 cells by PL-8 cells; B, Ac1-11[4Y] (100 ng/ml)-specific stimulation of 1934.4 cells in the presence of PL-8 cells. Data are representative of three experiments.

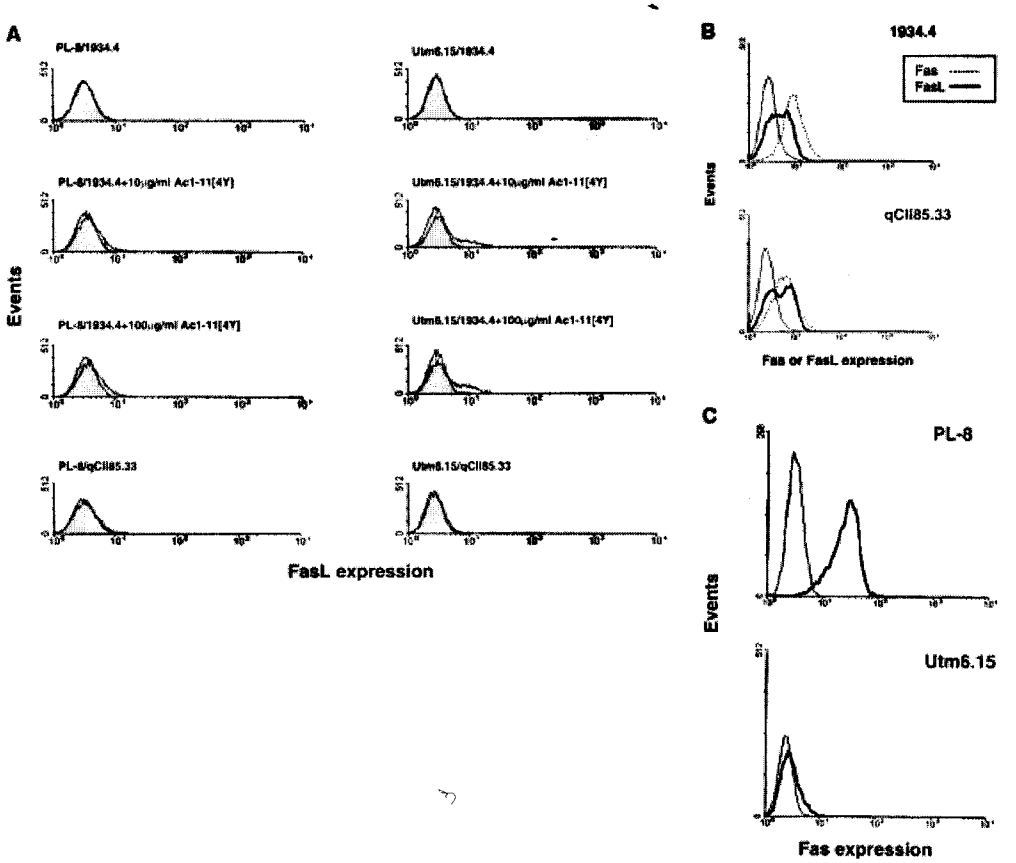


Fig. 4. Fas and FasL expression on T cell hybridomas. For A–C, Fas or FasL expression was detected using an anti-Fas or anti-FasL antibody (hamster derived) followed by biotinylated anti-hamster Ig and Extravidin-PE. A, 1934.4 T cells were stimulated with Ac1-11[4Y] in the presence of PL-8 or Utm6.15 as APC, and qCII85.33 cells were alloactivated with PL-8 or Utm6.15 cells without exogenously added peptide, for 4–5 h. FasL expression is shown by lines, and shaded histograms indicate fluorescence levels for cells incubated only with biotinylated anti-hamster Ig and Extravidin-PE. B, T cell hybridomas were activated with plate-bound anti-TCR β antibody F23.1 (10 μg/ml). Fas (dotted lines) and FasL (thick lines) expression levels are shown. Thin lines indicate fluorescence levels for cells incubated only with biotinylated anti-hamster Ig and Extravidin-PE. C, expression of Fas on PL-8 and Utm6.15 cells (thick lines). Thin lines indicate fluorescence levels for cells incubated only with biotinylated anti-hamster Ig and Extravidin-PE. Data are representative of two experiments.

ligand-stimulated 1934.4 cells did not induce cell death (data not shown).

2.3 The alloresponse is peptide dependent

The role of the MHC-bound peptide in allostimulation was studied by activating qCII85.33 with an I-A^u-transfected thymoma, Utm6.15, which expresses surface MHC class II levels comparable to those seen on PL-8 (analyzed by flow cytometry using three different anti-IA monoclonal antibodies, data not shown; [27]). There was no detectable IL-2 secretion by the qCII85.33 hybridoma in response to Utm6.15 cells, suggesting the requirement for a specific peptide for I-A^u-restricted allostimulation (Fig. 5 A). Utm6.15 cells have been previously shown to be very efficient in activating 1934.4 cells in a peptide-specific manner [27]. This indicates that I-A^u on these cells is functional, and consistent with the higher potency of Ac1-11[4Y] presentation relative to PL-8 cells, this cell line also induced higher expression levels of FasL on 1934.4 cells (Fig. 4 A).

The presence of the high-affinity I-A^u-binding peptide, Ac1-11[4Y] [25, 28], during *in vitro* stimulation did not affect IL-2 secretion by qCII85.33 cells following alloactivation. This suggests that the allopeptide might have a higher affinity for I-A^u. The peptide did not have any significant effect even when the PL-8 cell number was reduced tenfold (Fig. 5 B). The presence of Ac1-11[4Y]

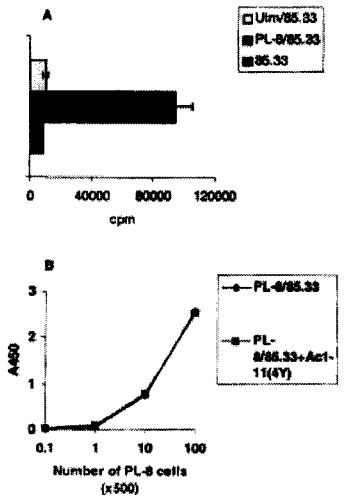


Fig. 5. A, allostimulation of qCII85.33 cells by I-A^u-expressing B cell lymphoma PL-8 or I-A^u-transfected thymoma cells, Utm6.15. 5×10^4 qCII85.33 cells were stimulated with 5×10^4 PL-8 or Utm6.15 cells for 20–24 h and IL-2 levels determined as described for Fig. 1. B, effect of high-affinity I-A^u-binding peptide Ac1-11[4Y] on alloactivation. 5×10^4 qCII85.33 cells were stimulated with various numbers of PL-8 cells with or without Ac1-11[4Y] (100 μg/ml) and IL-2 levels were analyzed 20–24 h later by sandwich ELISA [44]. Data are representative of three experiments.

during allostimulation also did not affect the lack of apoptosis of qCII85.33 cells in response to the alloligand (Fig. 2 B).

3 Discussion

The ability to recognize foreign antigens (as small peptides) in the context of self-MHC is acquired through positive selection during T cell development in the thymus [2–4]. This process also generates a large repertoire of alloreactive T cells which recognize foreign MHC molecules and form the basis of tissue rejection during transplantation [5]. Although recent studies have suggested that alloresponses are influenced by the degree of relatedness between the self-MHC and the MHC of the responding T cell [29], the molecular basis of alloreactivity has not been elucidated. In the present study we provide evidence in support of a peptide-dependent model of alloreactivity and demonstrate that subtle differences in the way an alloligand and a nominal peptide-MHC complex dock onto their TCR lead to differences in activation events. When complexed with I-A^u, the N-terminal epitope of MBP, Ac1-11, stimulated a specific T cell hybridoma to secrete IL-2 and undergo AICD. In contrast, allorecognition of I-A^u presented by the same B cell line resulted in high levels of IL-2 secretion by qCII85.33 hybridoma cells in the absence of significant cell death. The inability to undergo AICD following allostimulation was not due to an inherent defect in this T cell hybridoma, as activation with the anti-TCR β chain antibody (F23.1) efficiently induced expression of FasL and cell death. More importantly, we have previously shown that *in vitro* stimulation of qCII85.33 with the cognate ligand, recombinant (bovine) CII260-270:I-A^q complexes, resulted in significant apoptosis [24].

Further investigation revealed that the two TCR interact with different footprints on their ligands. A monoclonal antibody directed against an α chain determinant on I-A did not block IL-2 secretion by qCII85.33 cells in the alloresponse to PL-8, whereas it efficiently blocked Ac1-11:I-A^u induced IL-2 secretion and apoptosis in 1934.4 cells (Fig. 3; [24]). Significantly, both IL-2 secretion and induction of apoptosis in qCII85.33 cells following stimulation with the cognate ligand, CII260-270:I-A^q, are also efficiently blocked by Y3P [24]. Thus, the corresponding TCR-peptide-(self)MHC complexes might have similar footprints of interaction. In contrast, in the current analyses allorecognition and peptide-(self)I-A interactions appear to differ in the nature of the involvement of the I-A α chain. An antibody specific for the β chain of I-A blocked secretion of IL-2 by both 1934.4 and qCII85.33 cells following I-A^u-restricted peptide and I-A^u-restricted allostimulation, respectively. The data suggest that during allorecognition, the qCII85.33 TCR has either a minimal contact with the I-A^u α chain or it binds the I-A^u α chain at a site distinct from the 1934.4 TCR interaction site. The latter interaction site apparently overlaps with or is in proximity to the Y3P binding site. This difference between TCR-peptide-MHC configurations during the two types of responses apparently leads to poor induction of FasL expression on qCII85.33 cells during an alloresponse, although IL-2 secretion levels are unaffected. In turn, this results in almost insignificant AICD.

Many studies have shown a role for TCR-peptide-MHC affinity in affecting the outcome of the T cell-APC interaction [30–32]. In the current study, we are unable to determine the affinity of the relevant TCR-peptide-MHC interactions in the absence of identification of the allopeptide. However, the comparable levels of IL-2 secretion by qCII85.33 and 1934.4 cells following alloligand and MBP1-11[4Y]:I-A^u recognition, respectively, suggest that a difference in affinity/kinetics of the interactions does not account for the lack of apoptosis of qCII85.33 cells. More importantly, stimulation of qCII85.33 cells in ways that result in efficient apoptosis (F23.1 cross-linking; recombinant, plate-bound CII260-270:I-A^q complexes) induces similar levels of IL-2 as allostimulation [24]. Our data suggest that in addition to the affinity, the footprint

of a TCR-peptide-MHC interaction can have an effect on the quality of the T cell response.

The interaction of qCII85.33 cells with I-A^u alone was insufficient to induce alloreactivity, as the groove-bound peptide also plays a crucial role. This was demonstrated by the inability of an I-A^u-transfected thymoma, Utm6.15, to stimulate qCII85.33 cells. The expression of I-A^u on Utm6.15 cells was comparable with that seen on PL-8 cells. In addition, I-A^u on both cell types is recognized well by three different anti-I-A monoclonal antibodies (data not shown) ruling out any major conformational differences in I-A^u molecules expressed by the two cell lines. Furthermore, Utm6.15 cells act as potent APC for the I-A^u restricted MBP-specific T cell hybridoma 1934.4 [27], indicating that MHC class II on these cells is functional. The markedly different abilities of these two cell lines to activate qCII85.33 cells in an alloreactive fashion can thus be ascribed to variations in their peptide repertoires. The requirement for a specific peptide in the qCII85.33 alloresponse to I-A^u is consistent with the inability of recombinant Ac1-11[4Y]:I-A^u to activate this T cell hybridoma *in vitro* [24]. The Utm6.15 thymoma line therefore provides a useful tool to screen peptide eluates from PL-8 cells for the presence of the allopeptide.

The X-ray crystal structures of TCR-peptide-MHC complexes solved to date have shown that the TCR docks on cognate ligand in a diagonal orientation [6–10, 21, 33]. This has also been recently shown for an alloligand-TCR trimolecular complex [21]. It is probable that the qCII85.33 TCR-allopeptide-I-A^u interaction has a similar diagonal mode. Our data suggest that the limited apoptosis of qCII85.33 cells during an alloresponse to I-A^u results from reduced interaction between the I-A^u α chain and the TCR β chain. The TCR β chain plays an important role in shaping the T cell repertoire as it is involved in binding to endogenous (as well as exogenous) superantigens [34]. Binding of this chain of the TCR to superantigen-MHC complexes during T cell development results in T cell deletion through apoptosis. Conversely, inefficient engagement of the TCR β chain following ligation with a peptide-MHC complex could lead to reduced cell death, an effect that would be conducive to expansion of (alloreactive) T cells. Our results are consistent with previous studies demonstrating a more crucial role for the TCR α chain in ligand interaction during allorecognition than during nominal peptide-MHC recognition [35–37]. The lack of significant AICD during peripheral alloreactive responses is evident from a recent study in which inhibition of allorecognition by a human MHC class II derived peptide through the induction of apoptosis was reported [38]. The precise mechanism of this peptide-induced cell death is not clear but it is possible that the effect is mediated through efficient engagement of the TCR β chain by the peptide following binding to the MHC molecule.

Taken together, our study has revealed a mode and outcome of interaction for TCR alloligand binding that may represent an important mechanism for the generation of robust alloresponses. Further studies will be directed towards analyzing the generality of our results to other systems of allorecognition.

4 Materials and methods

4.1 Cell lines, antibodies and reagents

The I-A^u-expressing B cell line, PL-8, was generously provided by Dr. David Wraith, University of Bristol, GB [39]. An I-A^u-transfected derivative of BW4157, Utm6.15 [28, 40], was kindly made available by Dr. Harden McConnell, Stanford University, CA. An I-A^q-transfected derivative of the B cell lymphoma, M12.C3, was a generous gift of Dr. Edward Rosloniec (University of Tennessee, Memphis, TN). Hybridomas HB183 and TIB193 secreting the anti-I-A monoclonal antibodies Y3P and 10.2.16, respectively, and HB-32 secreting the anti-I-E monoclonal antibody 14.4.4S were obtained from the ATCC (Rockville, MD). The anti-Vβ monoclonal antibody F23.1 [41] was a generous gift of Drs. John Kappler and Philippa Marrack (University of Colorado Health Science Center). FITC-labeled H57-597 (anti-mouse TCR β), biotin-labeled anti-hamster IgG and antibodies to Fas and FasL were purchased from Pharmingen (San Diego, CA). FITC-labeled anti-mouse, anti-rat and anti-hamster IgG were obtained from Cappel. Extravidin-PE was purchased from Sigma Chemical Co. The N-terminal peptide (Ac1-11; acetylated at position 1) of rat MBP and an analog in which wild-type lysine at position 4 is substituted by alanine or tyrosine (Ac1-11[4A or 4Y]) were synthesized at the peptide synthesis unit of the Howard Hughes Medical Institute, UT Southwestern Medical Center, Dallas, TX. Enhanced chemiluminescence reagent (ECL) was purchased from Pierce. CTLL-2, an IL-2-dependent mouse T cell line, was provided by Dr. E. Vitetta (University of Texas Southwestern Medical Center, Dallas, TX).

4.2 T cell hybridomas

The murine T cell hybridoma 1934.4 is specific for the N-terminal peptide of MBP bound to I-A^u and was a generous gift of Dr. David Wraith (University of Bristol, Bristol, GB; [42]). The qCII85.33 T cell hybridoma recognizes bovine CII260-270 presented in the context of I-A^q and was kindly provided by Dr. Edward Rosloniec (University of Tennesse, Memphis, TN; [23]). These cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum.

4.3 T cell stimulation: analyses of IL-2 secretion and AICD

1934.4 T cells were incubated with various concentrations of the peptide Ac1-11 or position 4 analogs, Ac1-11 [4A or 4Y], in the presence of an I-A^u-expressing B cell lymphoma line, PL-8, or I-A^u-transfected thymoma line, Utm6.15, at 37 °C in a humidified CO_2 (5 %) incubator. qCII85.33 T cells were stimulated with APC, PL-8 or Utm6.15, without the addition of exogenous peptide. 20–24 h later, the supernatants were harvested and IL-2 levels determined using an IL-2-dependent cell line, CTLL-2 [43] or an ELISA as described previously [44].

For the analysis of AICD, T cells were activated with cognate ligands or plate-bound anti-Vβ monoclonal antibody F23.1 for 20–24 h. Cells were centrifuged, resuspended in 1 %BSA/PBS containing propidium iodide (PI, 10 μg/ml) and analyzed in a flow cytometer. Forward light scatter was plotted against PI fluorescence. Cells with high PI fluorescence were gated and are designated PI positive *i.e.* apoptotic [45], as viable cells have high forward scatter and low/no PI fluorescence.

4.4 Analysis of Fas and FasL expression

1934.4 and qCII85.33 T cells were stimulated with APC (PL-8 or Utm6.15) in the presence or absence of peptide or with plate-bound F23.1 for 4–5 h. The expression of Fas and FasL was determined by indirect immunofluorescence. Briefly, cells were washed and incubated with hamster anti-mouse Fas or FasL antibodies followed by biotinylated anti-hamster IgG and Extravidin-PE. Cells were analyzed using a flow cytometer (FACScan, Becton Dickinson, Mountain View, CA) and the program WinMDI2.8.

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