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Antigen presentation by mouse CD4+ T cells involving acquired MHC class II:peptide complexes: another mechanism to limit clonal expansion?

Running title: cell regulation by acquired MHC:peptide complexes

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Abstract

Antigen presentation by activated human and rat CD4+ T cells has long been known to induce hyporesponsiveness due to a combination of anergy and apoptosis. It has been assumed that no such phenomenon occurs in mice due to the inability of mouse T cells to synthesise MHC class II molecules. There have been several recent descriptions of the transfer of molecules, including MHC molecules, from antigen-presenting cells (APCs) to T cells. Here, we describe the acquisition of MHC class II molecules by TCR-transgenic T cells and T hybridoma cells following culture with APCs. Acquisition was markedly enhanced by T cell activation either due to cognate recognition of antigen or anti-CD3 activation. When activation was induced by antigen recognition preferential acquisition of complexes of class II molecules displaying cognate peptide was observed; in contrast, following activation by anti-CD3 the acquisition of class II molecules was MHC unrestricted. T cells that had acquired MHC class II:peptide complexes were able to act as APCs and induced proliferation and interleukin-2 secretion by resting T cells. However, when activated T cells that had acquired MHC class II:peptide complexes engaged in T:T interactions this led to an increase in apoptosis and the induction of hyporesponsiveness. These results raise the possibility that the acquisition of MHC class II:peptide complexes by T cells during an immune response may serve to limit clonal expansion, including that induced by alloantigen following tissue or stem cell transplantation.

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Introduction

MHC Class II molecules are constitutively expressed on specialised antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages. Antigen presentation by such cells to T cells results in T cell proliferation and differentiation towards effector function. However, MHC class II expression can be induced on multiple other cell types under the influence of cytokines such as gamma interferon (1, 2, 3). The consequences of antigen presentation by such non-specialised APC remain unclear. Most of the cell types on which MHC class II expression can be induced do not express the major co-stimulatory molecules CD80 and CD86. Using gamma interferon-treated primary cultures of human epithelial cells, we have previously reported the induction of antigen-specific unresponsiveness in human CD4+ T cells (2). In most species, T cells themselves express MHC class II molecules following activation (4, 5, 6). Antigen presentation by activated T cells to activated T cells has been reported by several groups to induce either T cell anergy or apoptosis (7, 8, 9, 10, 11, 12). Indeed, the first report of antigen-induced unresponsiveness resulted from the culture of a human T cell clone with its cognate peptide in the absence of added APC (7). Mechanistically this is a conundrum in that activated T cells also express substantial levels of B7 family co-stimulatory molecules (13, 14, 15). However, the regulatory effects of T:T antigen presentation may have a biological role in limiting clonal expansion at the later stages of an immune response. One species in which T cells do not synthesise MHC class II molecules is the mouse (16, 17). It has been assumed therefore that CD4+ T:T presentation does not apply in this species. However there have been several recent reports describing the acquisition of molecules by T cells from APC. These have included the transfer of MHC class I
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molecules (18, 19), B7 family molecules (20, 21) and MHC class II molecules in a series of rat experiments (22, 23, 24). The earliest description of MHC class II molecule transfer came from electron microscopy studies of thymocytes in irradiation chimaeras (25) and studies with T cell clones in vitro (26).

In this study we have examined the capacity of T cell receptor-transgenic and T hybridoma cells to acquire MHC II:peptide complexes from APC and subsequently present them to other T cells. The functional consequences of these events suggest that T:T antigen presentation may serve to limit T cell clonal expansion. The relevance of these findings to alloimmunity following tissue and stem cell transplantation is discussed.
Materials and Methods

Animals. TCR-transgenic DO11.10 mice were bred in the Biological Services Unit of the Imperial College Faculty of Medicine. BALB/c and CBA mice were obtained from Harlan.

Cell lines. The 3A9 murine CD4+ T cell hybridoma is specific for hen-egg white lysozyme (HEL) peptide (46-61) restricted by H2-Ak. A DO11.10 cell line was generated from DO11.10 TCR-transgenic mice and is specific for OVA peptide (323-339) restricted by H2-Ad. DAP.3-H2-Ak transfectants have been generated in our laboratory. CTLL2 is a murine IL-2 dependent CD8+ cell line for the detection of IL-2 production. T cells were maintained in RPMI 1640 supplemented with 10% FCS, 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 5 x10^-5 M 2-ME. DAP.3 transfectants were maintained in DMEM supplemented with 10% FCS, 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 5 x10^-5 M 2-ME with appropriate drug selection to maintain expression of the transfected genes.

Antibodies and flow cytometry. Antibodies used for the T cell analysis are listed: FITC-conjugated anti-H2-Ak (clone 14V.18; Serotec); cy-chrome conjugated DO11.10 TCR clonotypic antibody (clone KJ1-26, Caltag Lab); FITC-conjugated annexin V, FITC-conjugated CD44 (clone Ly-24), FITC-conjugated CD3 (clone 145-2C11), PE-conjugated anti-H2-Ak (clone 10.2.16), PE-conjugated anti-H2-Ad (clone AMS-32.1), PE-conjugated anti-TCR Vβ 8.2 (clone MR5-2), PE-conjugated anti-CD4 (clone GK1.5) and Cy-conjugated anti-CD4 (clone GK1.5) were obtained from Pharmingen. FITC-
conjugated sheep anti-mouse IgG was from Sigma. Anti-HEL (46-61)-H2-A^k complex (clone C4H3 – kindly provided by Caetano Reis e Souza) were purified from hybridoma culture (27). All flow cytometric analysis was conducted on a Becton Dickinson FACScalibur running CellQuest software.

**Generation of dendritic cells (DC).** The protocol of Inaba et al. (28) was used to generate DC from bone marrow. Briefly, bone marrow cells (5 x10^5 /ml) were cultured with 10% FCS RPMI 1640 containing 5% (v/v) supernatant from a GM-CSF-secreting transfected cell line. On day 3, non-adherent cells were removed. Fresh medium with GM-CSF was added to the adherent fraction for continuing culture. Cells were used on day 8 to day 10.

**Purification of CD4^+ T cells.** Lymph node and spleen cells were treated with a mixture of anti-CD8 and anti-H2-A^d supernatants (M5/114, YTS169 and YTS191) for 30 min. After antibody treatment, the cells were washed and incubated with anti-mouse and rat IgG dynal beads (Dynals) for 30 min. MHC Class II positive cells and CD8^+ T cells bound to the dynal beads were removed with a magnet. Purified CD4^+ T cells were recovered from the unbound cell suspension. To purify naïve CD4^+ T cells, anti-CD44 supernatant (Ly-24) was added to the antibody supernatant cocktail.

**In vivo stimulation of DO11.10 CD4^+ T cells.** 5x10^6 DO11.10 CD4^+ T cells were transferred into BALB/c mice. After 3 days, the mice were immunised either with CFA (Sigma) or CFA plus 300µg OVA peptide via foot pad injection. The DO11.10 CD4^+ T cells were recovered from draining lymph node 1 day after injection.
**T-APC co-culture and their subsequent separation.** APCs (DAP.3-H2-A^k^ transfectants or BALB/c DCs) were incubated with 5μM CFSE in PBS for 15 min in 37°C, and then washed with PBS three times. T cell responders (3A9 cells or DO11.10) were co-cultured with CFSE-labelled APC either pulsed with or without peptide overnight. CD4^+^ cells were isolated from APC by cell sorting and fixed with 1% (w/v) paraformaldehyde or γ-irradiated before being used as APC in T cell hybridoma or proliferation assays.

**T cell hybridoma assay.** 1x10^5^ fresh responder 3A9 cells were co-cultured with 2x10^5^ APC-preincubated 3A9 cells in 96 flat bottom microtitre plates. After 24 hours 50 μl supernatant was harvested and IL-2 activity determined by addition to the IL-2-dependent CTLL-2 cell line. CTLL-2 proliferation was measured by pulsing [^3^H] thymidine after a further 24 hours. Cells were harvested 18 hours later, followed by liquid scintillation spectroscopy. As a positive control, 5x10^4^ DAP.3-H2-A^k^ transfectants pulsed with HEL46-61 were used instead of fixed 3A9 APCs.

**T cell proliferation assays.** Responder CD4^+^ T cells (1x10^4^ cells/well) were stimulated with irradiated (30 Gray) APC (DC or APC-pre incubated T cells) in 96-well plates for 3 days. Proliferation was assessed by [^3^H] thymidine incorporation during the last 18 hours of 72 hour assays.

**Three stage culture for bi-directional T:T presentation in vitro.** DO11.10 CD4^+^ T cells were co-cultured with CFSE-labeled BALB/c DC with or without OVA peptide at a ratio of 4:1 for four hours to allow acquisition of MHC II or MHC II:peptide complexes from
DC. Afterwards, the T cells were purified from the DC by cell sorting. The purified DO11.10 CD4+ T cells that had either acquired H2-A^d:OVA complexes or H2-A^d alone were cultured in fresh medium overnight to allow T:T presentation to occur. DO11.10 CD4^+ T cells without co-culture served as a control. After the second stage culture, the cells were washed three times with PBS and then rested in fresh medium containing 10µg/ml of anti-H2-A^d (M5/114) for 5 days, to prevent any further cognate T:T interaction. After the rest culture, the cells were tested for proliferative responses to antigen restimulation by splenic APC.
Results

CD4+ T cells acquire substantial levels of MHC Class II molecules and MHC II:peptide complexes from antigen presenting cells

To explore MHC class II molecule acquisition by mouse CD4+ T cells, TCR-transgenic DO11.10 T cells were co-cultured with BALB/c dendritic cells with increasing concentrations of cognate peptide. After overnight culture the cells were harvested and stained with FITC-conjugated anti-CD4 and PE-conjugated anti-H2-A\textsuperscript{d} antibodies. The levels of H2-A\textsuperscript{d} expression on gated CD4+ T cells is shown in figure 1A. An antigen dose-dependent increase in MHC class II expression on the T cells was seen such that the majority of cells were MHC class II positive at the highest peptide concentration. These data indicate that the acquisition of MHC class II molecules was influenced by cognate recognition of the APC. However, significant MHC class II expression was also seen on the T cells co-cultured with DCs in the absence of added peptide. The kinetics of MHC class II acquisition is illustrated in figure 1B. As can be seen, readily detectable expression of H2-A\textsuperscript{d} on T cells was detectable within three hours of co-culture with the peptide-pulsed dendritic cells.

To further define the nature of the acquired MHC class II molecules we made use of a monoclonal antibody specific for a defined MHC II:peptide complex. This antibody, C4H3, is specific for a complex comprising H2-A\textsuperscript{k}:HEL\textsubscript{46-61}. The 3A9 murine CD4+ T cell hybridoma is specific for the same class II peptide complex. DAP.3-H2-A\textsuperscript{k} transfectants were pulsed with HEL peptide leading to expression of high levels of the C4H3 epitope (figure 1C). When the peptide-pulsed transfectants were incubated with
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3A9 cells overnight, the majority of 3A9 cells stained positively for H2-A\(^k\) expression and a significant proportion also displayed the C4H3 epitope (lower panels of figure 1C). In contrast when the 3A9 cells were cultured with the transfectants in the absence of peptide low level H2-A\(^k\) expression was detected and no staining was observed with C4H3. It is interesting to note that the fraction of H2-A\(^k\) molecules occupied by the HEL peptide was significantly increased on the 3A9 T cells, compared to the transfectant APC, as judged by the ratio of H2-A\(^k\) to C4H3 fluorescence (7.6 on the APCs versus 3.7 on the T cells). This suggests one of two possibilities, either the TCR contributes to the transfer by the physical capture of MHC II:peptide complexes or the transfer involves molecules that are enriched in the molecular synapse between T cell and APC such as the MHC II:peptide complexes for which the T cell is specific.

If the acquired MHC II:peptide complexes were to be of any functional significance \textit{in vivo} they would need to be stably expressed. The rate of decay of serologically detectable MHC class II molecules was assessed by culturing the sorted CD4\(^+\) T cells after MHC class II acquisition for varying time periods. As shown in Table 1 the percentage positive cells and the mean fluorescence intensity did decay over time. However readily detectable MHC class II expression was observed as much as 18 hrs after \textit{in vitro} culture.

**CD4\(^+\) T cells acquire MHC Class II molecules \textit{in vivo}.**

When staining freshly isolated ex-vivo CD4\(^+\) T cells from DO11.10 mice, we had consistently noticed a low level of staining with anti-MHC class II antibody which we
interpreted as non-specific background activity. However, having observed the above results we tested the possibility that this was genuine MHC II expression on the T cells due to acquisition in vivo. In order to address this, the T cells were either analysed immediately following purification or after 24 hrs of culture in the absence of APC. As shown in figure 2A, the percentage of positive cells and the mean fluorescence intensity both diminished after 24 hrs of culture suggesting that this was genuine MHC class II expression due to transfer of molecules in vivo.

To further characterise the freshly isolated ex vivo MHC II positive T cells, whole CD4+ T cells were purified from BALB/c mice and compared with the naive CD4+ cells prepared by depleting of CD44hi cells. The cells were then triple stained with anti-CD4, anti-H2-A^d and other markers. The H2-A^d+ CD4+ cells were found only in the whole CD4+ population but not in the naive population. They were also CD3+ confirming that they are CD4+ T cells. Moreover, they were confined to the activated/ memory population as shown by the anti-CD44 staining (figure 2B).

To examine whether activation can amplify the acquisition event in vivo, DO11.10 CD4+ T cells were transferred into BALB/c mice. After 3 days, the mice were immunised with CFA plus OVA peptide. Mice immunised with CFA or without any immunisation served as controls. For mice immunised with CFA plus peptide, DO11.10 CD4+ T cells showed higher expression of MHC class II as compared with the unimmunised animal, or animals immunised with CFA alone (figure 2C). This data indicates transfer of MHC class II can occur in vivo and is augmented by T cell activation.
MHC II molecule acquisition is markedly enhanced by T cell activation.

The mechanisms whereby T cells acquire molecules from APC are unclear. The levels of acquired complexes were markedly augmented following cognate recognition of MHC class II-presented antigen by APC, implying a role for the TCR in the capture of MHC II:peptide complexes. To distinguish between a direct involvement of TCR in the capture of complexes, and TCR-dependent T cell activation DO11.10 CD4+ T cells were co-cultured with BALB/c DCs or CBA DCs, in the presence or absence of OVA peptide or in the presence of anti-CD3 antibody. Low levels of MHC class II molecules were acquired by DO11.10 T cells irrespective of the types of DCs used (top panels of figure 3). This suggests that the cognate recognition independent mechanism of MHC class II capture is MHC unrestricted. However, addition of OVA peptide markedly increased acquisition of H2-A^d from BALB/c DCs, but made no difference to the levels of acquired H2-A^k, again suggesting the involvement of a cognate interaction. To determine whether the influence of cognate interaction reflected physical capture of complexes by the TCR or merely the need for T cell activation, anti-CD3 antibody was added to co-cultures of T cells with APC in the absence of antigen, or even with APC expressing irrelevant MHC class II molecules. In the presence of 1µg/ml of anti-CD3, DO11.10 T cells acquired comparable levels of MHC class II molecules from CBA DCs, as from BALB/c DCs in the presence of either peptide or anti-CD3. This makes it less likely that the TCR is physically involved in the capture of complexes. It is not clear whether the effect of T cell activation is mediated by some alteration in the properties of the T cell membrane that make it more acquisitive, or that T cell activation merely leads to increased intimacy of T
cell:APC conjugate formation. The enrichment of cognate MHC II:peptide complexes on the T cell surface following antigen-dependent activation, as seen with the 3A9 hybridoma, probably reflects the fact that transfer of molecules is concentrated at the interface between the T cell and the APC where cognate MHC II:peptide complexes are concentrated within the “molecular synapse” that is instrumental in T cell activation.

**MHC II:peptide complexes acquired by T cells can be recognised efficiently by other T cells.**

To investigate whether the acquired MHC II:peptide complexes on T cells can be presented to fresh responder T cells with the same antigen specificity, T cells were isolated from the T-APC co-cultures and used as APC in T cell hybridoma or T cell proliferation assays. Over 99% purity of isolated T cells was obtained after depletion of the CFSE-labelled APC by cell sorting as shown for DO11.10 T cells in figure 4D.

3A9 cells that acquired MHC II:peptide complexes (3A9\text{IIp}) were fixed and co-cultured with fresh responder 3A9 cells. The ability of 3A9 cells to present the acquired MHC II:peptide complexes to another T cell with the same antigen specificity was determined by their ability to induce IL-2 production by the fresh responder cells using the CTLL-2 bioassay. Figure 4A shows the positive control results of culturing 3A9 cells with the DAP.3 H2-A\text{k} transfectant as APC. This led to peptide-dependent IL-2 production. When 3A9\text{IIp} were used, comparable levels of IL-2 secretion were induced, albeit at a higher APC:T cell ratio (figure 4B). This demonstrates that 3A9 cells can not only acquire MHC
II:peptide complexes from APC, but the acquired complexes can be presented to other T cells with the same antigen specificity.

Parallel observations were made with T cell lines established from DO11.10 mice (specific for OVA_{323-339} ; H2A^d complexes) and peptide-pulsed BALB/c DCs. DO11.10 CD4^+ T cells were isolated from the T-DC co-culture using the method described above and used as APC. The cells were γ-irradiated and used to stimulate resting DO11.10 T cells. DO11.10 T cells that acquired the MHC II:peptide (DO11.10^{Hp}) isolated from co-cultures with peptide-pulsed BALB/c DCs stimulated proliferation of resting DO11.10 T cells (figure 4C) and the presentation could be blocked by adding anti-H2-A^d antibody (M5/114) (data not shown). Given the potency of dendritic cells as APCs, it was important to exclude the possibility that contaminating DC accounted for the observed proliferation. The level of DC contamination following T cell purification is shown in panel D of figure 4. A dose response was performed with DO11.10 T cells and DC numbers corresponding to 1% of the number of DO11.10^{Hp} cells used in the same experiment. The lower level of proliferation induced by this number of DC indicates that contamination is highly unlikely to account for the response induced by DO11.10^{Hp} T cells.

**Antigen presentation by T cells that acquired MHC II:peptide complex induces apoptosis and hyporesponsiveness.**

Human and rat data have demonstrated that T:T antigen presentation, involving activated T cells, induces anergy. To determine whether the presentation of acquired MHC
II:peptide complexes by activated mouse T cells to activated T cells would have a similar outcome, the DO11.10 T cell line (composed of activated T cells) was cultured with peptide-pulsed BALB/c DCs to allow MHC II acquisition, the DC were then removed and the T cells allowed to engage in T:T interactions. The T cells were then rested, by the addition of a blocking anti-H2-A^d antibody for five days. Finally, the T cells were re-challenged with peptide-pulsed BALB/c DCs. Annexin V staining of the T cells immediately after the period of T:T interaction revealed an increase in the fraction of the T cells undergoing apoptosis (15%) compared with the T cells exposed to DC antigen presentation (2%) (figure 5A). As shown in figure 5B, the cells that had engaged in T:T interactions were hyporesponsive compared with those had been cultured with DC, T cells that had acquired only MHC class II, or medium. The reduced proliferation could not be due to TCR:CD3 downregulation or premature T cell stimulation, because after five days resting, the levels of CD3:TCR had fully returned to normal and T cells that had been stimulated with DC had comparable proliferation to T cells in medium alone. The reduced proliferation was also accompanied by reduced IL-2 production (figure 5C) and hyperresponsiveness to exogenous IL-2 (figure 5D), a cardinal feature of T cell anergy.

Taken together these data suggest that mouse CD4^+ T cells acquire MHC II:peptide complexes from APC, can present these to each other, and that such T:T antigen presentation can induce both apoptosis and hyporesponsiveness.
Discussion

The first observation of T cell anergy was made when a human T cell clone was incubated with cognate peptide, in the absence of APC. The induction of anergy required the presentation of antigen by activated T cells to activated T cells. If activated human T cells were used as APC for resting human T cells, anergy did not occur, and strong proliferation was observed (Lombardi and Lechler, unpublished observations). Two considerations have cast doubt as to the likely significance of these findings; first, T cells are inefficient at antigen internalisation and processing, and second, mouse T cells do not synthesise MHC class II molecules, thereby challenging the generality of this phenomenon. The observations made here address these two issues.

There have been numerous reports of the transfer of molecules from APC to T cells, in mouse and rat systems. The first such observations were made using immuno-electron microscopic analysis of mouse thymic sections, in which murine thymocytes were noted to express MHC class II molecules, presumably acquired from thymic epithelium (25). More recently the capture of MHC class I and class II molecules by mouse and rat T cells from APC have been described (18, 19, 22, 23, 24). Additional reports of the transfer of costimulatory molecules from APC to T cells have been made (20, 21). Indeed, such acquired MHC:peptide complexes appeared to be functional, in that CD8+ T cells became sensitive to peptide-specific lysis by neighboring T cells after acquisition of MHC I:peptide complexes from APCs (18, 19). Also, naive T cells that had acquired CD80 from APC were capable of inducing IL-2 production by responder T cells (21).
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The results described extend these findings by showing that the presentation of acquired MHC II:peptide complexes has the same functional effects that have been observed using human T cells that express endogenously synthesised MHC class II molecules. In previous studies in a human system we have described heterogeneity amongst T cell clones, such that T:T antigen presentation by some clones induces apoptosis, while for other clones the dominant effect is the induction of anergy. Using a T cell line derived from DO11.10 TCR-transgenic mice, the presentation of acquired MHC class II:peptide complexes induced both apoptosis and anergy.

The mechanisms whereby T cells acquire molecules from APC are unclear. The levels of acquired complexes were markedly augmented by T cell activation. This was seen following cognate recognition of MHC class II-presented antigen by APC, implying a role for the TCR in the capture of MHC II:peptide complexes. However, the need for cognate recognition could be circumvented by the addition of anti-CD3 antibody to cocultures of T cells with APC in the absence of antigen, or even with APC expressing irrelevant MHC class II molecules. This makes it less likely that the TCR is physically involved in the capture of complexes. It is not clear whether the effect of T cell activation is mediated by some alteration in the properties of the T cell membrane that make it more acquisitive, or that T cell activation merely leads to increased intimacy of T cell:APC conjugate formation. Although the levels of MHC class II on T cells were increased in the context of activation, significant MHC class II acquisition was observed in the absence of activation. This activation-independent acquisition of MHC molecules appeared to be influenced by B7 family molecules, in that the acquisition of H2-Ad by
DO11.10 T cells from CHO transfectants was only observed, in the absence of antigen, when the transfectants co-expressed CD86 (data not shown). This result is consistent with the findings described previously by Sprent and colleagues (20), and may reflect the capacity of B7:CD28 interactions to induce intracellular signalling events, independently of TCR:CD3-transduced signals (29).

The in vivo significance of these observations remains a matter of speculation. We have argued previously that cognate interactions between T cells may provide an additional mechanism to limit T cell clonal expansion during the later stages of an immune response in lymphoid tissue. As T cells clonally expand, and move away from the DC that initiated their activation, they may engage in T:T interactions of the kind described here, thereby limiting the final clone size. The involvement of acquired complexes from the APC makes this a more credible mechanism of immunoregulation, in that it obviates the need for T cells to internalise and process soluble antigen that is likely to be in very limited supply in the lymph node. The fact that acquired complexes were readily detectable 18 hours after removal of the “donor” APC suggests that the acquired complexes may be sufficiently stable for T:T interactions to occur long after the T cell has moved away from the local APC. The detection of low level expression of MHC class II molecules on freshly isolated T cells ex vivo which decayed after 24 hours in culture and the amplification of these events in vivo following antigen-induced activation suggest that transfer of molecules from APC to T cell may indeed occur in vivo. The effects of T:T antigen presentation of acquired MHC:peptide complexes may play an important role in diminishing alloimmunity following haematological stem cell transplantation. Donor T
cells specific for minor or major histocompatibility antigens are likely to acquire MHC:antigen complexes from DCs. Presentation of these complexes to recently activated anti-recipient T cells may serve to diminish the anti-recipient alloresponse.

An alternative interpretation of these findings in an MHC class I-restricted system has been offered recently in the context of investigating competition between T cell populations with high and low T cell receptor affinity in vivo (30). In this study it was argued that the greater ability of the higher affinity T cells to remove MHC:peptide complexes from APC deprived the lower affinity T cells of adequate levels of cognate ligand to be activated. This was proposed as a mechanism of affinity maturation of T cells. Whichever interpretation is correct, the transfer of MHC:peptide complexes from APC to T cells appears to provide a further mechanism of immunoregulation, the nature of which will require elucidation by carefully designed in vivo experiments.
Acknowledgement

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References


Table 1. MHC class II molecules acquired by T cells are stable in culture.

3A9 cells were co-cultured with CFSE-labelled DAP3 H2-A\(^k\) pulsed with HEL\(_{46-61}\) peptide. To isolate 3A9 cells from the co-culture, 3A9 cells which were CFSE negative, were sorted. The sorted 3A9 cells, devoid of any APC, were then cultured in medium. The sorted 3A9 cells were stained simultaneously with cy-chrome-conjugated anti-CD4 and PE-conjugated anti-H2-A\(^k\) at different time intervals. The level of H2-A\(^k\) on 3A9 was analysis by flow cytometry. Only the CD4\(^+\) population was gated for analysis.

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3A9 control: MFI=5.15; %in M1 gate= 0.09
Figure legends

Figure 1 CD4⁺ T cells can acquire MHC II/peptide complexes from antigen presenting cells.

(A) CD4⁺ T cells purified from DO11.10 transgenic mice were co-cultured with bone-marrow derived BALB/c DC pulsed with different concentration of OVA323-339 peptide. After overnight co-culture, the cells were harvested and stained with PE conjugated anti-H2-Aᵈ and FITC-conjugated anti-CD4. The level of H2-Aᵈ expression on DO11.10 CD4⁺ T cells was measured by flow cytometry. Only CD4⁺ population was gated for analysis. [DO11.10 CD4⁺ T cells in medium control: MFI= 4.08; % in M1 gate = 3.04%]

(B) CD4⁺ T cells from DO11.10 transgenic mice were co-cultured with BALB/c DCs pulsed with 10µg/ml OVA peptide. Cells were harvested at different time points. The level of H2-Aᵈ on DO11.10 CD4⁺ cells is presented by histograms.

(C) 3A9 CD4⁺ T cell hybridoma were co-cultured with DAP.3-H2-Aᵏ transfectant prepulsed with or without 10µg/ml HEL46-61 peptide. The level of H2-Aᵏ expression and H2-Aᵏ:HEL₄₆-₆₁ complex on peptide pulsed DAP.3 transfectant were detected by staining with FITC conjugated anti-H2-Aᵏ or C4H3 monoclonal antibodies which is specific for H2-Aᵏ:HEL₄₆-₆₁ complex followed by FITC conjugated anti-mouse IgG. After overnight co-culture, 3A9 cells were harvested and stained for H2-Aᵏ and complex expression as mentioned, finally with PE-conjugated anti-CD4. The levels of H2-Aᵏ expression and C4H3 epitope on 3A9 cells were detected by flow cytometry. Only CD4⁺ population were gated for analysis. [DAP.3 control for C4H3 staining: MFI= 4.41, % in M1 gate = 1.29%; for H2-Ak staining: MFI = 3.44, % in M1 gate = 0.35%; 3A9 cells in medium
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control for C4H3 staining: MFI = 2.96; % in M1 gate = 0.66%; for H2-Ak staining: MFI = 3.23; % in M1 gate = 0.81%

Figure 2 CD4⁺ T cells can acquire MHC II in vivo.

(A) Freshly isolated CD4⁺ T cells from DO11.10 TCR-transgenic mice were stained with PE-conjugated anti-H2-A^d^ and FITC-conjugated anti-CD4 immediately following isolation or after 24 hours in medium. The level of H2-A^d^ on DO11.10 cells gated for CD4 expression is shown. These data have been reproduced in three animals. [MFI of whole CD4⁺ T cells: freshly isolated = 11.12; after 24 hr culture = 5.59]

(B) CD4⁺ T cells, either the whole preparation or purified naïve cells, from BALB/c mice were triple stained with PE-conjugated anti-H2-A^d^, cy-chrome-conjugated anti-CD4 and FITC-conjugated anti-CD3 or anti-CD44. Only the CD4⁺ population was gated for analysis.

(C) CD4⁺ T cells from DO11.10 transgenic mice were transferred to BALB/c mice. Some mice were immunised with CFA plus OVA peptide, or with CFA alone. T cells were recovered from the draining lymph node 1 day after immunisation. The cells were then triple stained with PE-conjugated anti-H2-A^d^, FITC-conjugated anti-CD4 and cy-chrome-conjugated DO11.10 TCR clonotypic antibody (KJ1-26). Three mice were included in each group. CD4⁺ KJ1-26⁺ and CD4⁺ KJ1-26⁻ cells were analysed seperately as indicated on the figure. Representative mice from each group are shown.

Figure 3 MHC class II molecule acquisition is marked enhanced by T cell activation.
DO11.10 CD4+ T cells were co-cultured overnight with bone marrow derived BALB/c DCs or CBA DCs either without peptide, with 10µg/ml OVA<sub>323-339</sub> peptide or 1µg/ml soluble anti-CD3. Cells were then harvested and stained with PE-conjugated anti-H2-A<sup>d</sup> or anti-H2-A<sup>k</sup>, and FITC-conjugated anti-CD4. The levels of H2-A<sup>d</sup> and H2-A<sup>k</sup> on DO11.10 CD4+ cells were analysed by flow cytometry. Only the CD4+ population was gated for analysis. [DO11.10 CD4+ T cells in medium control for H2-A<sup>d</sup> staining: MFI= 10.64; % in M1 gate = 1.66%; H2-A<sup>k</sup> staining: MFI= 18.76; % in M1 gate = 3.22%]

Figure 4 The acquired MHC II/peptide complexes by T cells can be recognised by fresh responder T cells with the same antigen specificity.

(A) DAP.3-H2-A<sup>k</sup> transfectant either pulsed with [DAP3(H2-A<sup>k</sup>/HEL)] or without [DAP3(H2-A<sup>k</sup>)] HEL<sub>46-61</sub> were used to stimulate IL-2 production by fresh 3A9 responder cells.

(B) 3A9 cells were co-cultured with CFSE-labelled DAP.3 H2-A<sup>k</sup> transfectants pulsed with or with HEL<sub>46-61</sub> peptide. 3A9 populations that had acquired H2-A<sup>k</sup> [3A9<sup>II</sup>] or H2-A<sup>k</sup>:HEL<sub>46-61</sub> complexes [3A9<sup>IIIp</sup>] and 3A9 cells cultured in medium only [3A9] were isolated as above, fixed, and used to stimulate IL-2 production by fresh responder cells. 2x10<sup>5</sup> fixed 3A9-APCs were co-cultured with fresh 3A9 responders. After 24 hours incubation, 50 µl of supernatant was harvested. IL-2 activity in the supernatant was measured by CTLL2 bioassay.

(C) DO11.10 CD4+ T cells were co-cultured with CFSE-labelled BALB/c DCs that has been pulsed either with or without OVA<sub>323-339</sub> peptide to allow MHC class II acquisition. After overnight co-culture CFSE negative DO11.10 CD4+ T cells were sorted and used as
APCs. The CFSE negative DO11.10 CD4⁺ T cell populations that had acquired H2-A<sup>d</sup> [− □ −] or H2-A<sup>d</sup>/OVA<sub>323-339</sub> complexes [− ▲ −] were γ-irradiated to stimulate proliferation of fresh responder cells. DO11.10 CD4⁺ T cells cultured in medium only [− ● −] and peptide-pulsed DC [− ○ −], cell numbers corresponding to 1% of the number of DO11.10<sup>Hp</sup> cells used in the same experiment, were used as controls. (D) The purity of the isolated cells was assessed by flow cytometry.

Figure 5 Bi-directional T:T presentation by the T cells that acquired the MHC II/peptide complexes can induce T cell hyporesponsiveness and apoptosis.

DO11.10 CD4⁺ T cells after co-culture with BALB/c DCs either pulsed with or without OVA<sub>323-339</sub> peptide were purified after 4 hours co-culture. The purified CD4⁺ T cells that had acquired H2-A<sup>d</sup> [CD4<sup>II</sup>] or H2-A<sup>d</sup>:OVA<sub>323-339</sub> complexes [CD4<sup>Hp</sup>] were continue cultured for 24 hours to allow them to engage in T:T interaction. (A) Some cells were harvested and stained with Annexin V and propidium iodide to test for apoptosis. DO11.10 CD4⁺ T cells cultured before in medium only [CD4] and DO11.10 CD4⁺ T cells cultured with OVA<sub>323-339</sub> peptide-pulsed BALB/c DCs for 24 hours [CD4-DC/p] were used as controls. The remaining cells were then rested in the presence of blocking anti-H2-A<sup>d</sup> antibody for five days. (B) The proliferative response to antigen rechallenge with BALB/c DCs was measured by T cell proliferation. [Background cpm: CD4= 208.7, CD4<sup>II</sup>= 269.7, CD4<sup>Hp</sup>= 5619.2, CD4-DC/p= 6027.1] and IL-2 production by the DO11.10 CD4⁺ T cells upon antigen rechallenge was measured by CTLL2 bioassay. [Background cpm: CD4= 726.3, CD4<sup>II</sup>= 1191.9, CD4<sup>Hp</sup>= 7203.1, CD4-DC/p= 2986.8] (D) The
response of the DO11.10 CD4+ T cells to exogenous IL-2 was measured by T cell proliferation assay.
FIGURE 1

A

DO11.10 CD4 + BALB/c

<table>
<thead>
<tr>
<th>Peptide dose (µg/ml)</th>
<th>Counts</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12.74</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>27.70</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>43.67</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>57.93</td>
<td></td>
</tr>
</tbody>
</table>

B

Time after co-cultured with 10 µg/ml OVA peptide pulsed BALB/c DC

<table>
<thead>
<tr>
<th>Time</th>
<th>MFI</th>
<th>% in M1 gate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
<td>11.12</td>
<td>5.44</td>
</tr>
<tr>
<td>3 hr</td>
<td>72.02</td>
<td>45.39</td>
</tr>
<tr>
<td>20 hr</td>
<td>96.51</td>
<td>73.17</td>
</tr>
</tbody>
</table>

C

Peptide pulsed DAP3-H2-A^k

<table>
<thead>
<tr>
<th>Peptide</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A9</td>
<td>72.11</td>
</tr>
<tr>
<td>3A9 + DAP3-H2-A^k</td>
<td>98.17</td>
</tr>
</tbody>
</table>

3A9 + peptide pulsed DAP3-H2-A^k

<table>
<thead>
<tr>
<th>Peptide</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A9</td>
<td>0.53%</td>
</tr>
<tr>
<td>3A9 + DAP3-H2-A^k</td>
<td>19.69%</td>
</tr>
</tbody>
</table>

MHC II/peptide complex

H2-A^k
FIGURE 2

A Freshly isolated

<table>
<thead>
<tr>
<th>M1</th>
<th>5.44%</th>
<th>M1 MFI = 181.15</th>
</tr>
</thead>
</table>

B Whole CD4

<table>
<thead>
<tr>
<th>M1</th>
<th>2.80%</th>
<th>M1 MFI = 26.53</th>
</tr>
</thead>
</table>

| H2-Ad     |       |                 |

| Control   |       |                 |

| CD44      |       |                 |

C Naive CD4

<table>
<thead>
<tr>
<th>M1</th>
<th>0.63%</th>
<th>M1 MFI = 26.53</th>
</tr>
</thead>
</table>

D Control

<table>
<thead>
<tr>
<th>M1</th>
<th>2.38%</th>
<th>M1 MFI = 26.53</th>
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</thead>
</table>

E CFA

<table>
<thead>
<tr>
<th>M1</th>
<th>2.38%</th>
<th>M1 MFI = 26.53</th>
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</thead>
</table>

F CFA+ OVA

<table>
<thead>
<tr>
<th>M1</th>
<th>0.63%</th>
<th>M1 MFI = 26.53</th>
</tr>
</thead>
</table>

G H2-Ad

|       |       |                 |

H Whole CD4

<table>
<thead>
<tr>
<th>M1</th>
<th>0.63%</th>
<th>M1 MFI = 26.53</th>
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</table>

I Naive CD4

<table>
<thead>
<tr>
<th>M1</th>
<th>2.38%</th>
<th>M1 MFI = 26.53</th>
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</table>

J Control

<table>
<thead>
<tr>
<th>M1</th>
<th>2.38%</th>
<th>M1 MFI = 26.53</th>
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</table>

K CFA

<table>
<thead>
<tr>
<th>M1</th>
<th>0.63%</th>
<th>M1 MFI = 26.53</th>
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</thead>
</table>

L CFA+ OVA

<table>
<thead>
<tr>
<th>M1</th>
<th>2.38%</th>
<th>M1 MFI = 26.53</th>
</tr>
</thead>
</table>
FIGURE 3

DC only

DC and 10µg/ml OVA peptide

DC and 1µg/ml soluble anti-CD3 antibody

BALB/c DC

CBA

MFI=20.90

MFI=31.60

MFI=95.11

MFI=39.72

MFI=94.03

MFI=92.69

20.12%

76.61%

20.92%

50.85%

12.45%

40.26%

50.85%

12.45%

40.26%

50.85%

12.45%

40.26%
FIGURE 4

A

DAP.3-H2-A\(^{k}\)

B

3A9

C

DO11.10 CD4

D

<1% DC contamination

For personal use only.
FIGURE 5

A

B

C

D
Antigen presentation by mouse CD4+ T cells involving acquired MHC class II:peptide complexes: another mechanism to limit clonal expansion?

Julia Y S Tsang, Jian-Guo Chai and Robert Lechler