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Thymocyte positive selection results in maturation to the single-positive stage, while negative selection results in death by apoptosis. Although kinetic analyses indicate only 3–5% of CD4^+ thymocytes reach the single-positive stage, the balance of positive and negative selection and the nature and quantity of cells mediating maximal negative selection are uncertain. Here, using a system where the number and type of stromal cells and thymocytes can be controlled, we investigated the maturation of CD4^+ thymocytes in the presence or absence of thymic epithelium and dendritic cells (DC) from wild-type (wt) and H-2M^−/− mice expressing different peptide arrays. We find that titration of wt DC into reaggregates of wt epithelium has a dramatic effect on the number of CD4^+ cells generated, with 1% DC causing a maximal 80% reduction. Moreover, while addition of 1% wt DC into cultures of H-2M^−/− epithelium causes a 90% reduction in CD4^+ cells, no effect was observed when similar numbers of wt thymic epithelium were added. Collectively, these data provide the first accurate indication of the quantity and quality of stromal cells required for maximal negative selection in the thymus, demonstrate the importance of peptide diversity in T cell selection, and highlight a large degree of overlap between positive and negative selection events. The Journal of Immunology, 1998, 161: 6599–6603.

Selection of the αβ TCR repertoire is essential for the production of a T cell population that is capable of efficient recognition of self MHC molecules presenting nonself peptides but that is nonreactive to the same MHC molecules presenting self peptides (1, 2). This process of repertoire selection takes place at the CD4^+ stage of T cell development in the thymus and involves positive selection, triggering further maturation to the single-positive CD4^+ and CD8^+ stages (3) and negative selection that induces programmed cell death in potentially autoreactive thymocytes (4).

A key factor in determining whether a CD4^+ thymocyte undergoes positive or negative selection is the avidity of its TCR-mediated interaction with peptide/MHC complexes on thymic stromal cells (5, 6). CD4^+ thymocytes are known to have a finite lifespan of 3–4 days in the thymic cortex, during which time successive αβ TCR combinations, generated by ongoing TCR α-chain gene rearrangements (7), are tested for interaction with stromal peptide/MHC ligands. Those cells that fail to interact with stromal MHC complexes during this time die from neglect (8), while low avidity interactions lead to positive selection and survival, and high avidity interactions lead to negative selection through apoptosis. Recent evidence suggests that there is a considerable degree of promiscuity in TCR-mediated peptide/MHC recognition, such that a single or a limited array of peptides can positively select cells that are able to recognize a range of other peptides presented by the same MHC molecule (9–12). However, although varied, the diversity of such repertoires is limited, indicating that peptide diversity is important in generating a fully diverse TCR repertoire during positive selection (13–15).

In addition to TCR ligation of the appropriate avidity, there is also evidence that other factors are involved in determining the outcome of thymic selection. Thus, efficient positive selection is dependent upon interaction with peptide/MHC complexes on thymic cortical epithelial cells (16), while negative selection is most efficiently mediated by professional APCs of bone marrow origin, namely dendritic cells (DC) (17, 18). At present, it is not clear whether this cellular specialization for selection involves: differential expression of MHC-bound peptide arrays on positively or negatively selecting cells, the expression of a specialized peptide repertoire on positively selecting cells, or differential expression of costimulatory molecules that provide additional signals leading to either cell differentiation or cell death (19).

In this study, we explore the interaction of peptide diversity and selecting cell type in T cell selection events, using reaggregate thymus organ cultures (RTOCs) to recombine thymic epithelial cells and DC from wild-type (wt) or H-2M^−/− mice with a restricted presentation of MHC class II-bound peptides (9–11). Our results show that when any influence of negative selection by DC is excluded, positive selection by epithelial cells bearing a limited MHC class II-bound peptide spectrum is reduced threefold compared with positive selection by wt epithelium. Importantly, this is not due to differences in MHC class II levels on H-2M^−/− and wt epithelial cells, and thus provides direct evidence for the role of peptide diversity in the efficiency of positive selection by thymic epithelial cells. Moreover, we have quantitated the frequency of bone marrow-derived DC required to induce maximal negative selection of T cell repertoires generated on both wt and H-2M^−/− epithelial cells. Remarkably, we find that the number of CD4^+ cells generated by wt epithelium is reduced by 80% in the presence of small numbers of wt DC (1%), while the generation of CD4^+ cells in the presence of H-2M^−/− epithelium is even further reduced (by 95%) by the same number of wt DC, emphasizing the

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3 Abbreviations used in this paper: DC, dendritic cells; RTOC, reaggregate thymus organ culture; wt, wild type; PE, phycoerythrin.

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impacts of peptide diversity in negative selection in relation to that in positive selection. Finally, we show that in direct comparison on a per cell basis, wt thymic epithelial cells and wt DC, with similar overall levels of MHC class II expression, differ markedly in their ability to induce negative selection in a population of cells positively selected on a limited array of peptides, arguing that these two cell types are qualitatively different in the signals they provide for negative as well as positive selection.

Materials and Methods

Mice

H-2M<sup>−/−</sup> mice, H-2<sup>b</sup> haplotype (10), obtained from The Jackson Laboratories (Bar Harbor, ME), were bred and maintained at the Biomedical Services Unit, University of Birmingham. Embryos from these and wt C57BL6 (H-2<sup>b</sup> haplotype) were obtained by timed matings, with the day of detection of the vaginal plug being designated as day zero.

Abs and immunoconjugates

Anti-rat and anti-mouse IgG-coated Dynabeads (Dynal, Wirral, U.K.) were coated with the following Abs, as appropriate: anti-CD3 (clone KT3; a kind gift of Dr. Julian Dyson, Medical Research Council Clinical Sciences Centre, London, U.K.), anti-CD8 (clone YTS 169.4; Sera Lab, Sussex, U.K.), anti-CD45 (clone M19; American Type Culture Collection, Manassas, VA), anti-I<sup>α</sup>A<sup>β</sup> (clone AF6–120.1; PharMingen, San Diego, CA), anti-DEC205 (clone NLDC-145; Serotec, Oxford, U.K.), and anti-CD11c (clone M1/9; American Type Culture Collection, Manassas, VA). Anti-rat and anti-mouse IgG-coated Dynabeads (Dynal, Wirral, U.K.) were used for negative as well as positive selection.

Cell separations

Preparation of thymocytes. CD4<sup>+</sup> thymocytes at a preselection stage of development were prepared from newborn thymus cell suspensions by immunomagnetic separation, exactly as described (16, 17). Such a procedure results in a population of preselection CD4<sup>+</sup>8<sup>+</sup> TCR<sup>+</sup> thymocytes at ≥99% purity (20). Isolation of thymic stromal cells. Thymuses from wt and H-2M<sup>−/−</sup> 15-day mouse embryos were cultured in 2-deoxyguanosine for 5–7 days and trypsinized to form a single-cell suspension, as described (16, 17). After depletion of residual hemopoietic elements with anti-CD45-coated Dynabeads, CD11c<sup>+</sup> thymic epithelial cells (Ref. 16, 17, and 20, and data not shown). Isolation of thymic DC. DC were isolated from the pooled axial, inguinal, and brachial lymph nodes of 4- to 6-wk-old wt and H-2M<sup>−/−</sup> mice by immunomagnetic selection. Briefly, lymph node suspensions were subject to positive selection with anti-I<sup>α</sup>A<sup>β</sup>-coated magnetic beads, followed by further positive enrichment of DC using beads coated with anti-DEC205. Beads were then removed by pronase treatment, as described (21).

Formation of RTOCs

Freshly prepared thymocytes and appropriate stromal cells were mixed together in 1.5 ml Eppendorf tubes (Laser, Southampton, U.K.) at the necessary ratios and pelleted by centrifugation. Following removal of the supernatant, the cell pellet was carefully transferred to the surface of a 0.8 µm Nucleopore filter (Corning Costar U.K., High Wycombe, U.K.) in organ culture. Under such conditions, intact thymus lobes reform from these mixtures within 12–18 h (16, 17, 20).

Flow cytometric analysis

Thymocytes harvested from RTOCs and freshly isolated stromal cells were analyzed using a dual-laser Epics Elite machine (Coulter, Hialeah, FL) with forward and side scatter gates set so as to exclude nonviable cells, as described (17).

Results

Effect of peptide diversity on the efficiency of positive selection

Recent studies have suggested that when positive selection is confined to a limited array of peptide/MHC class II complexes, the number of single-positive CD4<sup>+</sup> cells and the diversity of repertoire generated are reduced compared with that seen in selection in a wt environment (14, 15). However, these studies are based on models in which the selecting ligands are expressed on both positively (thymic epithelial cells) and negatively (thymic DC of bone marrow origin) selecting cells, so that the overall outcome of selection reflects the balance of these two processes. Thus, while positive selection on a limited peptide set may be highly efficient, this could be reduced by exposure to the same set of peptides on cells mediating negative selection.

Using RTOCs, when purified CD4<sup>+</sup>8<sup>+</sup> thymocytes can be exposed only to a single defined stromal cell type (cortical epithelial cells), it is possible to look at the efficiency of positive selection mediated by these cells in the absence of any influence from DC. Therefore, we compared the efficiency of CD4<sup>+</sup> cell generation from purified CD4<sup>+</sup>8<sup>+</sup> precursors with wt, as compared with H-2M<sup>−/−</sup>, thymic epithelium in the absence of DC. RTOCs with equivalent numbers of CD4<sup>+</sup>8<sup>+</sup> thymocytes and either wt (H-2<sup>b</sup>) or H-2M<sup>−/−</sup> (H-2<sup>h</sup>) epithelial cells were prepared and harvested after 5 days. Thymocyte yields were determined and positive selection assessed by flow cytometric analysis of the number of CD4<sup>+</sup>8<sup>+</sup> and CD4<sup>+</sup>8<sup>+</sup> cells.

Fig. 1, a and c, shows that in RTOCs, as expected (20), CD4<sup>+</sup>8<sup>+</sup> and CD4<sup>+</sup>8<sup>+</sup> cells are generated in the presence of wt epithelium at a ratio of 3:1. However, in RTOCs initiated with H-2M<sup>−/−</sup> thymic epithelial cells, although normal numbers of CD4<sup>+</sup>8<sup>+</sup> cells are generated (Fig. 1c), there is a marked reduction in the development of CD4<sup>+</sup>8<sup>+</sup> cells, with a ratio between CD4<sup>+</sup>8<sup>+</sup> and CD4<sup>+</sup>8<sup>+</sup> cells being ~1:1 (Fig. 1b), and an overall
While CLIP peptide expression is predominant on H-2M<sup>m</sup> molecules are occupied by CLIP peptides in wt epithelium (Fig. 2). plexes using mAb 30-2 (22) showed that only a fraction of class II and a<sup>CD4</sup>pared RTOCs of wt epithelium, defined numbers of preselection cells mediating this effect were not defined. Therefore, we pre-

...duction of H-2M<sup>c</sup> on H-2M<sup>m</sup> and H-2M<sup>+</sup>(f) cells with forward/side scatter characteristics of DCs, gating on CD11c<sup>low</sup> cells. Negative controls were set up using an irrelevant isotype matched Ab.

**FIGURE 2.** Comparison of expression of TCR ligands on thymic epithelial cells and DC. Freshly trypsinized 2-dGuo-treated thymus lobes from wt and H-2M<sup>−/−</sup> embryos were analyzed for expression of I-A molecules (a and b) and CLIP/I-A complexes with the mAb 30–2 (c and d). Analysis of I-A levels on lymph node DC was performed by two-color labeling of lymph node suspensions for I-A<sup>β</sup> PE and CD11c FITC. Histograms shown are for wt (e) and H-2M<sup>−/−</sup> (f) cells with forward/side scatter characteristics of DCs, gating on CD11c<sup>low</sup> cells. Negative controls were set up using an irrelevant isotype matched Ab.

A fourfold reduction in the number of CD4<sup>+</sup>8<sup>−</sup> cells (Fig. 1c). Importantly, this reduction in CD4<sup>+</sup>8<sup>−</sup> numbers generated by H-2M<sup>−/−</sup> epithelial cells is not due to lower levels of MHC class II expression, since flow cytometric analysis showed MHC class II levels to be comparable on wt and H-2M<sup>−/−</sup> epithelium (Fig. 2, a and b). Interestingly, expression of class II molecules is occupied by CLIP peptides in wt epithelium (Fig. 2c), while CLIP peptide expression is predominant on H-2M<sup>−/−</sup> thymic epithelial cells (Fig. 2d). These data exclude the possibility that the reduced generation of CD4<sup>+</sup> cells in H-2M<sup>−/−</sup> mice is due to the impact of negative selection by DC with a peptide/MHC class II profile comparable to that on the epithelial cells mediating positive selection. Thus, we have obtained direct evidence that peptide diversity plays a key role in the efficiency of CD4<sup>+</sup> T cell selection by thymic epithelium.

**Differential effects of peptide diversity in negative selection**

Having established a system whereby positive selection on thymic epithelium takes place unopposed by negative selection by DC, we were able to examine the effects of DC-mediated negative selection on the positively selected repertoire. Thus, we next analyzed the impact of negative selection by DC on the generation of positively selected CD4<sup>+</sup> cells, both in terms of the efficiency of the deleting cells on a per cell basis and the effects of differences in peptide repertoire between cells mediating positive and negative selection events.

In an initial series of experiments, we first established the optimal number of DC required to mediate negative selection. Previous studies using chimeric organ cultures (23) have suggested that nonepithelial hemopoietic elements are efficient mediators of negative selection, although the precise identity and number of cells mediating this effect were not defined. Therefore, we prepared RTOCs of wt epithelium, defined numbers of preselection CD4<sup>+</sup>8<sup>−</sup> thymocytes, and titrated numbers of either purified wt or H-2M<sup>−/−</sup> DC. Cultures were harvested after 5 days and examined for the effects of DC number on generation of CD4<sup>+</sup> cells. As shown in Fig. 3, the addition of wt DC to RTOCs with wt epithelial cells has a considerable impact on the number of single-positive CD4<sup>+</sup> cells generated. This effect reached a plateau in the presence of 1% DC, when the yield of CD4<sup>+</sup> cells was reduced by ~80%. These observations confirm the potency of DC as mediators of negative selection and imply that even when DC represent as few as 1% of the total cell number per reaggregate culture, they can make contact with the majority of thymocytes. Such findings agree well with the relative scarcity of DC in the thymus as compared with thymic epithelial cells (8, 17). They also show that more than two-thirds of the repertoire positively selectable by wt epithelial cells is susceptible to removal by negative selection. Previous estimates of the effects of negative selection on the positively selected repertoire have ranged considerably (8, 12, 24–26), from as little as 5% (24) to as much as 50% (25). The results presented here clearly provide further evidence for a high degree of overlap between T cell selection processes.

In marked contrast to the effects seen with wt DC, H-2M<sup>−/−</sup> DC expressing a limited array of MHC class II bound peptides had a much less dramatic effect on the number of CD4<sup>+</sup> cells that were produced in the presence of wt epithelium, causing a reduction of only 20% even when added at a frequency of 10% of total cell number. This excludes the possibility that the extensive deletion seen with wt cells is due to the nonspecific effects of incorporating DC into RTOC. Importantly, overall levels of MHC class II expression on wt and H-2M<sup>−/−</sup> DC were found to be the same (Fig. 2, e and f), indicating that the magnitude of deletion seen with wt DC is a reflection of the wider spectrum of peptides presented by the MHC class II molecules on those cells. Conversely, the reduced effect of H-2M<sup>−/−</sup> DC suggests that, although peptide recognition in thymocyte selection may be promiscuous to a degree (14, 15), this is not sufficient to allow a limited array of peptides to have a predominant effect via negative selection on a repertoire positively selected on a wider range of peptides. When RTOCs were prepared in which positive selection occurred on H-2M<sup>−/−</sup> epithelium, addition of wt DC caused an even
more dramatic reduction (95%) in the number of single-positive CD4+ cells generated than that seen when positive selection occurred on wt epithelium (Fig. 4). This dramatic reduction, which is comparable to in vivo chimeric experiments (14), is unlikely to be solely due to the recognition of CLIP peptides on the wt DC, since H-2M<sup>−/−</sup> DC (expressing predominantly CLIP/MHC class II complexes) have a less profound effect on the generation of H-2M<sup>−/−</sup>-selected CD4<sup>+</sup> cells (Fig. 4). Thus, the most likely explanation for these findings is that many of the cells selected on peptide/self MHC complexes on H-2M<sup>−/−</sup> epithelial cells have sufficiently high TCR-mediated avidity for other peptide/self MHC complexes present on wt DC to trigger negative selection. This is consistent with previous observations that T cells selected on H-2M<sup>−/−</sup> epithelium and allowed to reach maturity display a considerable degree of “self” reactivity to homozygous wt DC in proliferation assays (9–11). On this note, it may well be the case that CD4<sup>+</sup> thymocytes positively selected by thymic epithelial cells in the absence of DC, show a degree of reactivity to self peptide/MHC complexes, since these cells would be negatively selected if allowed to mature in the presence of DC. We are currently testing this possibility in additional experiments.

**Efficiency of different stromal cell types in mediating negative selection**

Although a role for DC in negative selection is well established, the ability of epithelial cells to mediate negative selection is still controversial (18, 27). The major impact of DC expressing a spectrum of wt peptides on the limited T cell repertoire positively selected on MHC class II/limited peptide complexes in H-2M<sup>−/−</sup> mice demonstrated in the previous section provided us with an ideal model with which to compare the efficiency of DC and epithelial cells in mediating negative selection. For this purpose, RTOCs were prepared from H-2M<sup>−/−</sup> epithelial cells and preselection CD4<sup>+</sup>8<sup>+</sup> thymocytes with the further addition of either 1% wt DC or 1% wt thymic epithelial cells. As shown in Fig. 5b, while wt DC caused a reduction in the numbers of CD4<sup>+</sup>8<sup>+</sup> thymocytes, and both mature CD4<sup>+</sup>8<sup>+</sup> and CD4<sup>+</sup>8<sup>+</sup> subsets, a similar number of wt epithelial cells had no impact on the CD4<sup>+</sup>8<sup>+</sup> population or the generation of CD4<sup>+</sup>8<sup>+</sup> cells (Fig. 5c). Again, this is unlikely to be due to differences in the overall level of MHC class II molecules on wt epithelium and wt DC, since these two cell types were found to display comparable MHC class II levels in flow cytometric analysis (Fig. 2, a and e). These data therefore argue that thymic epithelial cells and DC possess other qualitative differences that underlie their different abilities in mediating positive and negative selection.

**Discussion**

In this study, we have provided direct evidence that diversity of peptide presentation by MHC class II molecules on thymic epithelial cells increases the efficiency with which CD4<sup>+</sup> cells are positively selected. We also show that much of this selectable repertoire is potentially autoreactive and is susceptible to negative selection. This effect is particularly dramatic when positive selection is mediated by epithelial cells expressing MHC class II molecules bearing a limited array of peptides, and negative selection is mediated by wt DC presenting a more diverse peptide array. Thus, consistent with the reported promiscuity of TCR-mediated peptide/MHC recognition (9–12), there appears to be considerable overlap between positive and negative selection, even when they are mediated by different peptide arrays. Moreover, these effects imply that confining positive selection to a limited array of specialized peptides could place severe constraints on repertoire diversity. However, it should be noted that even when MHC class II molecules are predominantly loaded with a single peptide, as in the case of H-2M<sup>−/−</sup> mice and the CLIP peptide, Grubin et al. (28) have shown that other MHC class II-bound peptides are present in these mice that play a direct role in positive selection of CD4<sup>+</sup> thymocytes. Indeed, in agreement with the findings of Grubin et al. (28), our analysis of CLIP expression on MHC class II<sup>+</sup> thymic epithelial cells from H-2M<sup>−/−</sup> mice shows that some of these cells do not express CLIP (Fig. 2). Thus, while our data support the notion that peptide diversity bound to MHC class II molecules on thymic
epithelial cells plays a key role in the efficiency of positive selection, the degree of positive selection mediated by CLIP and other non-CLIP peptides is uncertain.

Our findings also address the issue of stromal cell specialization for positive and negative selection. The ability of small numbers of DC (i.e., no more than 1% of total cell number per reaggregate lobe) to delete a substantial proportion of the CD4+ cells selectable on thymic epithelium emphasizes the efficiency of these cells as mediators of negative selection.

In marked contrast to the ability of small numbers of wt DC to cause a dramatic reduction in CD4+ cells capable of positive selection on H-2M1−/− epithelium, addition of the same number of wt epithelial cells had no discernible effect. Since the overall levels of MHC class II molecules expressed on wt DC and wt epithelium were found to be similar (Fig. 2), this argues that there are other qualitative differences underlying the differing ability of these cells to mediate negative selection. These differences may be overridden where avidity dependent on TCR alone is high, as in the case of some TCR transgenic mice with higher than normal levels of TCR expression on CD4+ thymocytes, especially where high levels of cognate peptide were also present. Such factors may explain the reported ability of thymic epithelium, albeit from adult as compared with our fetal sources, to mediate negative selection in some models (18). However, within the range of TCR expression on normal thymocytes, as used in this study, differences in the ability to provide costimulatory signals may be one factor in determining the differential efficiency of epithelium and DC in mediating negative selection. In this regard, it is interesting that B7 expression is absent from thymic epithelial cells but present on thymic DC (29), especially as its ligand, CD28, is expressed on cortical thymocytes, and B7/CD28 costimulation has been shown to influence negative selection (30, 31). Similarly, differential expression of cell adhesion molecules affecting the overall avidity of T cell/stromal cell interaction may be important in determining outcomes of selection and will be an area for further study.

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