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Analysis of APC Types Involved in CD4 Tolerance and Regulatory T Cell Generation Using Reaggregated Thymic Organ Cultures

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Tolerance to self-Ags is generated in the thymus. Both epithelial and hematopoietic thymic stromal cells play an active and essential role in this process. However, the role of each of the various stromal cell types remains unresolved. To our knowledge, we describe the first comparative analysis of several types of thymic hematopoietic stromal cells (THSCs) for their ability to induce CD4 tolerance to self, in parallel with the thymic epithelium. The THSCs—two types of conventional dendritic cells (cDCs), plasmacytoid dendritic cells, macrophages (MΦs), B lymphocytes, and eosinophils—were first characterized and quantified in adult mouse thymus. They were then examined in reaggregated thymic organ cultures containing mixtures of monoclonal and polyclonal thymocytes. This thymocyte mixture allows for the analysis of Ag-specific events while avoiding the extreme skewing frequently seen in purely monoclonal systems. Our data indicate that thymic epithelium alone is capable of promoting self-tolerance by eliminating autoreactive CD4 single-positive thymocytes and by supporting regulatory T cell (Treg) development. We also show that both non-Treg CD4 single-positive thymocytes and Tregs are efficiently deleted by the two populations of cDCs present in the thymus, as well as to a lesser extent by MΦs. Plasmacytoid dendritic cells, B lymphocytes, and eosinophils were not able to do so. Finally, cDCs were also the most efficient THSCs at supporting Treg development in the thymus, suggesting that although they may share some characteristics required for negative selection with MΦs, they do not share those required for the support of Treg development, making cDCs a unique cell subset in the thymus. The Journal of Immunology, 2013, 190: 000–000.

How immunological tolerance to self is maintained became a central question very early in immunology (1). It is now well known that the main events in generating self-tolerance occur in the thymus. There, immature T cells reactive against self-Ags are deleted in a process called negative selection (2). Although the removal of autoreactive cells is an essential process, it is not complete. Hence, the thymus also produces regulatory T cells (Tregs) that are capable of regulating the remaining, potentially self-reactive cells outside the thymus (3). The developmental outcome for any individual T cell, ranging from deletion to lineage diversion (4, 5), depends on many factors that are not fully understood.

The thymic stroma has an active and essential role in the development of the diverse T cell populations in the thymus. “Stroma” includes epithelium and a number of hematopoietic cell populations. In this article we refer to the latter cell types as thymic hematopoietic stromal cells (THSCs). The thymic epithelium can be divided into two phenotypically and functionally distinct cell populations: cortical thymic epithelial cells and medullary thymic epithelial cells (mTECs). Although cortical epithelial cells are necessary for producing a functional self-MHC–restricted T cell repertoire through positive selection (6), mTECs are directly and indirectly (7, 8) associated with the removal of self-reacting T cells through negative selection. Among THSCs, dendritic cells (DCs) are predominantly located in the medullary region (9) but are also present in the cortex (10, 11). They have long been associated with clonal deletion (8, 12–14) and more recently with Treg development (9, 14, 15). Three types of DCs have been identified in the thymus (16, 17). Plasmacytoid DCs (pDCs), which develop in the periphery and migrate to the thymus, were shown to play a role in Treg induction in the human thymus (18, 19). Recirculating conventional DCs (cDCs), which also develop outside the thymus, can directly present Ags from the periphery and delete developing thymocytes reactive to them (14, 20, 21). It is not clear whether they are also able to take up and present thymic Ags. A second type of conventional DC (cDC) develops within the thymus (cDCs) from a common DC–T cell precursor (22). These cells can present thymic Ags expressed by themselves or picked up from the surroundings, as well

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as peripheral self-Ags picked up from mTECs that express the AIRE transcription factor (8). In addition to DCs, other THSCs, such as macrophages (MΦs), eosinophils (Eos), and B lymphocytes (LBs), are present in the thymus. Thymic MΦs have long been associated with the clearance of apoptotic thymocytes (23, 24). Little is known about the role of Eos or LBs in the thymus. The former were associated with apoptotic thymocyte clearance (25, 26), whereas it was suggested that B cells play a role in negative selection of CD4+ CD8- single-positive (SP4) but not CD8+ CD4- single-positive (SP8) thymocytes (27).

Several approaches have been used to determine the role of the various thymic cell populations in the induction of tolerance. Seminal studies by Sprét, von Bohmer, and other investigators (28–31) addressed the relative role of epithelium versus bone marrow-derived cells in the induction of tolerance using bone marrow chimera and thymus grafts. They used functional measures of tolerance, and the results varied widely depending on the details of the system used. However, those studies were based on tolerance to major MHC alloantigens, which may differ from that to minor MHC Ags. Indeed, it was shown by von Bohmer and Hafen (32) that thymic epithelium was able to induce CD8 tolerance to minor, but not major, histocompatibility Ags, highlighting the relevance of the type of Ag to the functional outcome.

More recently, the identification of Tregs and the use of genetically modified models, such as MHC-deficient or TCR-transgenic mice, have facilitated the study of the induction of tolerance to peptide Ags. However, a consensus on the specific role of each thymic APC has not been reached. As examples, although some studies found that the thymic epithelium was able to only partially delete both SP4 and SP8 autoreactive thymocytes when expressing a model Ag (33), others found it capable of fully deleting autoreactive SP4 (7), or else autoreactive SP8 but not SP4 (8). Further, hematopoietic cells were shown to delete SP4 and, to a lesser extent, SP8 in MHC-deficient bone marrow chimeras (13); SP4 and SP8 on cognate Ag picked up from mTECs (8); or autoreactive SP4, but not SP8, in a similar system (33) and in mice depleted of CD11c+ cells (34). Although clonal deletion of autoreactive thymocytes has primarily been associated with the medullary compartment of the thymus, it was also shown to be driven by DCs in the thymic cortex in mice (35). Indirect evidence suggests that this may also be the case in the human thymus (36).

Thymic-derived Tregs were shown to be enriched in autoreactive cells (37). However, the factors that determine whether autoreactive T cells are deleted or converted to Tregs remain a matter of debate (4). Although most Foxp3+ cells are found among the SP4 thymocytes in the medulla, the thymic compartment in which Treg lineage commitment actually takes place is not clear (38, 39). Both thymic cortex and medulla were found to be suitable sites for Treg lineage commitment (39–42). Current models for Treg development highlight either instructional control (external signals that drive the T cell lineage choice) or stochastic processes (T cell–intrinsic decisions) (4, 43). In the context of the former, both thymic epithelial cells (42, 44) and DCs (9, 15) were found to support Treg development. However, it was also proposed that no dedicated APC is necessary but that any APC could provide the required TCR stimulus, because Treg differentiation would depend mainly on a T cell–intrinsic developmental window of susceptibility (45). Overall, despite significant progress in our understanding of the establishment of self-tolerance, the role of each type of thymic APC in this process remains unresolved.

In this study, we performed, to our knowledge, the first comparative analysis of the role of six types of THSC (pDCs, cDCrecs, cDCThys, MΦs, LBs, and Eos) and the thymic epithelium in the establishment of self-tolerance in the CD4+ T cell lineage. We used reaggregated thymic organ culture (RTOC), a technique originally established by Anderson et al. (6, 46), to individually test the function of each APC type. This system closely mimics the thymus in three-dimensional structure and ability to support T cell development. We show that both the thymic epithelium and the two populations of thymic cDCs were capable of eliminating autoreactive SP4 thymocytes, as well as supporting Treg development; pDCs, LBs, and Eos were not able to do so. Further, we show that MΦs were also able to delete SP4 but failed to efficiently support Treg development. This highlights a qualitative functional difference between DCs and MΦs and indicates the existence of a requirement, in addition to a strong TCR signal, for Treg induction but not negative selection.

**Materials and Methods**

**Mice**

C57BL/6 CD45.2 mice were purchased from Charles River and housed at the Centre d’Exploitation et de Recherche Fonctionnelle Experimentale (Evry, France) for programmed mating. At 14 and 15 d of pregnancy, females were sacrificed to obtain fetal thymus. OT-II are TCR-transgenic mice that express a Vβ5Vα2 TCR recognizing a peptide consisting of aa 323–339 of OVA (OVAp) in the context of I-A<sup>b</sup> (47). OT-II RAG2<sup>−/−</sup>CD45.2<sup>+</sup> and B6 CD45.1 mice were housed and bred at the Institut Curie in an accredited specific pathogen-free colony. Live animal experiments were done in accordance with the guidelines of the French Veterinary Department.

**THSC purification**

Single-cell suspensions of thymic stromal cells were isolated as previously described (48). Briefly, 16 thymus from 8–10-wk-old B6 mice were pooled, cleaned of nonthymic tissue, and cut in pieces. The fragments were digested with 0.125% (w/v) collagenase D and 0.1% (w/v) DNase I (both from Roche) in CO₂-independent (Life Technologies) medium supplemented with 10% FCS, 100 μg/ml streptomycin, and 100 μg/ml penicillin (Invitrogen), at 37°C with gentle agitation over 30 min. The remaining fragments were dissociated with gentle pipetting. The cell suspension was filtered (70 μm mesh) and washed with fresh medium. Cells were counted and incubated with FcR-blocking Ab (clone 2.4G2 supernatant) prior to staining with anti-CD11c and anti-CD19–coated magnetic microbeads (Miltenyi Biotec), according to the manufacturer’s protocol. The cell suspension was run on an autoMACS Pro (Miltenyi Biotec) using the “Posseled” program. The purity of the positive fraction was ~95%. The positive fraction was recovered, counted, and stained with the relevant Abs for FACs sorting of the six THSC populations. The yield of THSCs obtained was 0.32 ± 0.15% (of 14 independent purifications) from total thymus digests of 3.5 ± 1 × 10<sup>6</sup> cells.

**Immunofluorescent staining for flow cytometry**

mAbs conjugated with the following fluorochromes (FTC, PE, PerCP, PE-Cy5, PE-TR, PE-Cy7, allophycocyanin, Alexa Fluor 700, allophycocyanin-Cy7, eFluor 450) or biotinylated Abs revealed with a conjugated streptavidin (all purchased from BD Pharmingen, E Bioscience, and BioLegend) were used for flow cytometry, according to standard techniques, on a LSR II (Becton Dickinson) or FACSDiva cell sorter (BD Biosciences). DAPI and excitation at 405 nm were used for the exclusion of dead cells when the staining was not intracellular. An FcR-blocking Ab (clone 2.4G2 supernatant) was used prior to Ab staining. The following Abs were used for sorting and analyzing: CD45.1 (A20), CD45.2 (104), TCRβ (H57-597), CD24 (HS4; M1/69), CD8α (Ly-2), CD4 (L3T4), B220 (CD45R; RA3-6B2), CD19 (I-D3), CD11c (HL-3), CD11b (M1/70), F4/80 (B8), Sirpsy (CD172a; P64), H2<sup>K<sub>B</sub></sup> (MHC I; AF6-88.5), IA-IE (MHC II; M5/114.15.2), CD40 (3/23), CD50 (16-10A1), CD86 (GL-1), Siglec-F (E50-2440), EpCAM (CD326; clone G8.8), CD25 (PC61), and intracellular Foxp3 (FJK-16s). Intracellular staining was performed according to the manufacturer’s specifications (eBiosciences).

**Cell sorting**

The cell suspensions were sorted on a FACSDiva cell sorter (BD Biosciences) at ~8 × 10<sup>6</sup> events/s and 20 p.s.i. Samples were collected in pure FCS and analyzed for purity. CD4<sup>+</sup> CD8<sup>+</sup> double-positive (DP) thymocytes and THSC populations were obtained with purities generally >99%, with the exception of MΦs, which usually ranged between 90 and 95%. DP B6 and OT-II thymocytes were positively sorted using anti-CD11c and anti-CD8 Abs. For the sorting of the six THSC populations from the CD11c<sup>+</sup> and...
CD19-enriched fraction, their characteristic cell surface marker combination was used for positive gating, whereas the other Abs in the mixture were used for negative gating for higher purity.

**RTOCs**

RTOCs were prepared as described elsewhere (6, 12). Briefly, three categories of cells were mixed together and carefully deposited in a standing drop to form reaggregated tissue: thymic stroma (mainly thymic epithelium), immature thymocytes, and a particular THSC where indicated.

The thymic stroma was obtained from E14/15 B6 fetal thymus that had been treated with 2-deoxyguanosine (dGuo; Sigma; 1.35 mM for 6 d) and further depleted of remaining CD45+ cells to eliminate all hematopoietic cells. After dGuo treatment, the fetal thymus were washed extensively in fresh medium and digested with 0.05% trypsin, 0.02% EDTA at 37°C for 25 min, followed by pipetting until complete digestion. The digest was stained with anti-CD45–coated magnetic microbeads (Miltenyi Biotec), according to the manufacturer’s protocols. The cells were run on an autoMACS Pro (Miltenyi Biotec) using the “deplete” program using the “deplete” program. The negative fraction was recovered and counted.

DP thymocytes were obtained from 2-3-wk-old B6 or OT-II mice. Total thymic cell suspensions were obtained by mechanical disruption of the thymus. Cells were counted, stained with the described Abs, and sorted by FACS.

Purified THSCs were loaded with OVAp by incubation in a 10 μg/ml (5.7 μM) OVAp solution at 37°C for 30 min for the majority of experiments or with varying OVAp concentrations, as indicated. Cells were washed by centrifuging them through a 100 μl serum cushion.

The fetal thymic stroma (epithelium) was mixed with the sorted thymocytes at a 1:3 ratio, and a single THSC population per condition was added where indicated at 0.1% of total number of cells. The cell mixtures were aliquoted at 1 × 10^6 cells into 1.5 ml Eppendorf tubes. Tubes were centrifuged at 350 × g, and the supernatants were completely removed. The pellets were dispersed into a slurry by careful mixing and finally were gently delivered as a standing drop on a filter. RTOCs were cultured on Millipore membranes for 5 d of culture. The thymic stroma alone was capable of promoting the development of OT-II thymocytes and supporting Treg development. For this, we performed a set of RTOCs that included thymic epithelium and a 50/50 mixture of monoclonal and polyclonal immature thymocytes (OT-II/B6 RTOCs) in the presence of varying doses of the cognate Ag (Fig. 2A). Although 50% of thymocytes being monoclonal is clearly a much higher frequency than expected for any clonal thymocyte in vivo, it does allow for the analysis of Ag-specific events. The presence of 50% polyclonal thymocytes provides an internal control for T cell development and ensures a proper thymic structure (49). The addition of a graded dose of the cognate peptide also elicits Treg development by providing enough niches to prevent the intraclonal competition that would otherwise limit Treg development (50). To ensure the complete absence of hematopoietic cells in the input cell preparation, thymic epithelium was obtained from dGuo-treated fetal thymic lobes that were further depleted of any remaining CD45+ cells (Supplemental Fig. 2A). Polyclonal and monomolecualr immature (DP) thymocytes were obtained from B6 and OT-II RAG-deficient (OT-II) mice, respectively. The use of purified CD4+CD8+ DP thymocytes ensured the removal of mature SP thymocytes potentially capable of responding to Ag through activation and proliferation. Further, the corresponding absence of early CD4+CD8− double negative (DN) precursors prevented the de novo development of DCs or Møs in the cultures. Varying doses of OVAp (OT-II’s cognate Ag) were added to the culture medium. After 5 d of culture, all DP thymocytes, both B6 and OT-II, had matured into SP cells (see Supplemental Fig. 2B for thymocyte maturation and gating strategy).

Increased Ag concentration led to a reduction and eventual elimination of OT-II SP4 cells (Fig. 2B, 2C), suggesting that negative selection by the epithelium alone may occur. However, there was a concomitant and unexpected appearance of OT-II mature DN and SP8 cells, suggesting either a deviation from the normal development of OT-II thymocytes or an increased survival of aberrant T cells (expressing an MHCII-restricted TCR and a nonmatching coreceptor). Note that the input thymocytes in these RTOCs were immature (βTCR CD24+ β) and that after 5 d of culture they had matured (βTCR CD24−) into DN (51, 52) and CD8 T cells. This deviation and/or increased survival may be occurring either in parallel with or instead of deletion.

Further analysis of the OT-II SP4 population showed that Treg (SP4 Foxp3+) development was dependent on the presence and dose of the cognate Ag (Fig. 2B, 2D). This shows that the thymic epithelium alone can support Treg development and that a strong TCR stimulus is required. The analysis of Treg development according to Ag dose showed that, at the lowest Ag concentrations at which Tregs first develop (0.08 μg/ml OVAp, Fig. 2B, lower

**Results**

**Characterization of THSCs**

To assess the role of various THSC types in the establishment of self-tolerance, we first purified and characterized these populations. Total digests of adult B6 thymi were enriched for cells expressing CD11c or CD19 and subsequently analyzed for the expression of a number of relevant markers (Fig. 1A, 1B). Established markers for APCs, such as MHC class II (MHCII) and costimulatory molecules (CD40, CD80 and CD86), show the highest levels on the two DC populations (cDCrecs and cDCthys) and intermediate levels on Møs and B cells. pDCs expressed very low or no detectable levels of MHCII molecules, although a low level of expression was reported previously (16). Eos were negative for MHCII, in agreement with previous reports (25, 26).

A parallel analysis in the spleen showed the existence of six similar cell populations (Supplemental Fig. 1), with the exception of cDCrecs, which showed greater expression of MHCII and costimulatory molecules in the thymus than in the spleen, in agreement with a previous report (17).

The proportion of THSCs that we obtained from digests of total adult thymus was 0.32 ± 0.15% (mean ± SD) of 14 independent purifications. Nevertheless, this number is most likely an underestimate of the real proportion of THSCs in the thymus, because there is probably significant loss of the cells of interest with the purification method used in this study. This is in agreement with a published estimate, which reported that ~0.5% of the total number of cells in an adult thymus are DCs (16).

The relative abundance of each of the studied THSC populations is also shown in Fig. 1A. Among these six purified THSC populations, B cells were the most abundant, followed by the two cDC populations and then Eos. Møs and pDCs were quite rare.

**The thymic epithelium alone is able to promote tolerance to self**

First, we set out to study the contribution of thymic epithelial cells in the establishment of self-tolerance and found that thymic epithelium alone is capable of both eliminating autoreactive SP4 thymocytes and supporting Treg development. For this, we performed a set of RTOCs that included thymic epithelium and a 50/50 mixture of monoclonal and polyclonal immature thymocytes (OT-II/B6 RTOCs) in the presence of varying doses of the cognate Ag (Fig. 2A). Although 50% of thymocytes being monoclonal is clearly a much higher frequency than expected for any clonal thymocyte in vivo, it does allow for the analysis of Ag-specific events. The presence of 50% polyclonal thymocytes provides an internal control for T cell development and ensures a proper thymic structure (49). The addition of a graded dose of the cognate peptide also elicits Treg development by providing enough niches to prevent the intraclonal competition that would otherwise limit Treg development (50). To ensure the complete absence of hematopoietic cells in the input cell preparation, thymic epithelium was obtained from dGuo-treated fetal thymic lobes that were further depleted of any remaining CD45+ cells (Supplemental Fig. 2A). Polyclonal and monomolecular immature (DP) thymocytes were obtained from B6 and OT-II RAG-deficient (OT-II) mice, respectively. The use of purified CD4+CD8+ DP thymocytes ensured the removal of mature SP thymocytes potentially capable of responding to Ag through activation and proliferation. Further, the corresponding absence of early CD4+CD8− double negative (DN) precursors prevented the de novo development of DCs or Møs in the cultures. Varying doses of OVAp (OT-II’s cognate Ag) were added to the culture medium. After 5 d of culture, all DP thymocytes, both B6 and OT-II, had matured into SP cells (see Supplemental Fig. 2B for thymocyte maturation and gating strategy).

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Establishment of self-tolerance and found that both cDCs and M\textsubscript{F}s, but not other THSCs, negatively select SP4 cells and Tregs.

We next investigated the role of various THSC types in the establishment of self-tolerance and found that both cDCs and M\textsubscript{F}s, but no other THSCs, were able to delete SP4 thymocytes and Tregs. To do this, we used the six purified THSC populations to supplement RTOCs consisting of thymic epithelium and polyclonal immature thymocytes (Fig. 3A). To reproduce a normal thymic context as closely as possible, the THSC populations were added individually to the RTOCs at 0.1% of the total number of cells (0.016–0.08 \mu g/ml OVAp). This suggests that a stronger TCR signal is required for deletion than for Treg induction. Further, when clonal deletion first becomes apparent, as indicated by the reduction in the absolute numbers of non-Treg SP4 cells (0.08–0.4 \mu g/ml OVAp, Fig. 2B, upper panels), the number of Tregs is preserved (0.08–0.4 \mu g/ml OVAp, Fig. 2B, lower panels). This supports the notion of Tregs being more resistant to negative selection than non-Treg SP4 cells. Finally, at even higher doses (0.4–2 \mu g/ml OVAp, Fig. 2B, lower panels), there was a reduction in both the absolute numbers of Tregs and their proportion among SP4 cells (Fig. 2D). Although the reduction in the absolute numbers of Tregs may be explained by the elimination of SP4 cells, the reduction in the proportion of Tregs among SP4 cells suggests either deletion or a failure to develop.

Overall, these results show that the thymic epithelium alone is capable of imparting tolerance to self that may include deletion of autoreactive SP4 thymocytes. Further, in the complete absence of THSCs, the thymic epithelium is sufficient to support Treg development, albeit inefficiently, as shown by the low number of cells generated in these experimental settings.

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After 5 d of culture, all DP thymocytes had matured into SP cells (Fig. 3B, upper panels), and Tregs had developed (Fig. 3B, lower panels). The absolute numbers of non-Treg (Foxp3\textsuperscript{−}) SP4 cells and Tregs in each condition were compared with the respective numbers in cultures with no added THSC (epithelium alone) (Fig. 3C). A reduced number of thymocytes in a given condition, compared with epithelium alone, was interpreted as negative selection by the added THSC population, whereas an increased number was interpreted as indicating developmental support.

In these RTOCs, addition of either of the two cDC subsets induced a strong decrease in the numbers of both non-Treg SP4 cells and Tregs, suggesting the occurrence of negative selection (Fig. 3C). The same effect, although not as strong, was observed when adding M\textsubscript{F}s. The addition of other THSC types to the cultures did not show any significant effect on thymocyte numbers. These data suggest that cDCs and M\textsubscript{F}s are able to negatively select non-Treg SP4 cells, as well as Tregs. Although the overall effect when adding cDCs clearly indicates deletion, this outcome could still result from a combination of both induction and negative selection of Tregs. Therefore, the role of THSCs in Treg development was investigated further in an Ag-specific system.

cDCs are the most efficient THSCs in supporting Treg development.

Next, we studied the role of the various THSC types in Treg development and found that cDCs were the most efficient APC types for supporting Treg development. For this, we supplemented OT-II/ B6 RTOCs with purified, OVAp-loaded THSC subpopulations (Fig. 4A). Note that no OVAp was added to the culture medium, allowing a normal development of OT-II thymocytes into SP4 cells (Fig. 4B).

Unexpectedly, we observed a reduction in the number of OT-II SP4 cells when the RTOCs were supplemented with OVAp-loaded Eos (Fig. 4C). This was not observed when adding untreated Eos to the polyclonal B6 RTOCs (Fig. 3C), and given the lack of...
MHCII expression on Eos, it seemed unlikely that the effect could be explained by an Ag-specific negative-selection process. Control experiments using either untreated or mock-loaded Eos demonstrated that the reduced number of SP4 cells found above was due to some nonspecific Eo activation or damage during the loading and washing treatment and was independent of the presence of Ag (Supplemental Fig. 3).

Analysis of the THSC-supplemented RTOCs showed that the two OV Ap-loaded cDC populations were the most efficient at negatively selecting OT-II thymocytes (Fig. 4C). MΦs could also delete, but to a lesser extent, whereas the other THSCs were unable to do so. This pattern was consistent with that observed on B6 thymocytes, present either on their own in polyclonal RTOCs (Fig. 3) or developing alongside OT-II cells (Supplemental Fig. 4A), indicating that there is no fundamental difference between the two culture systems. In no case did the addition of an OV Ap-loaded THSC produce an increased development of mature DN or SP8 cells (Fig. 4B, upper panels) as was seen with the epithelium alone (Fig. 2B, upper panels). The viability of the six THSC populations after the 5-d culture could not be assessed given their small number.

cDCs were by far the most efficient cells at supporting Treg development (Fig. 4D). Although MΦs showed some variability between experiments with regard to the Treg proportion present in these cultures (Fig. 4D, left panel), this was not reflected in the absolute numbers (Fig. 4D, right panel).

Finally, to assess the specificity of the observed effects, we performed an Ag dose-response titration using OT-II/B6 RTOCs supplemented with cDCthys that had been loaded with increasing concentrations of OVAp (Fig. 5). In these RTOCs, OT-II Treg development was dependent on the presence of the cognate Ag and proportional to its concentration. There was also no negative selection observed on OT-II thymocytes when the supplementing cDCthys carried no OVAp (Supplemental Fig. 4B).

Overall, these results show that cDCs are the most efficient THSC type at negatively selecting autoreactive T cells, as well as at supporting Treg development (Table I). MΦs were also able to delete autoreactive T cells, although to a lesser extent, but they were only weak supporters of Treg development.

**Discussion**

In this work, we first characterized and quantified six distinct THSC populations for the expression of a variety of surface molecules, including a number of hallmark APC markers, as well as their cytology in adult mouse thymus. These populations were then examined for their ability to induce self-tolerance using RTOCs. As in a normal thymus, the thymic epithelium is essential to ensure a three-dimensional architecture and positive selection within RTOCs (6). Therefore, the ability of the thymic epithelium alone to induce self-tolerance was studied in cultures containing purified DP immature thymocytes to avoid activation and proliferation from mature SP thymocytes to the Ag.

**FIGURE 2.** The thymic epithelium can induce tolerance to self. RTOCs were made of fetal thymic stroma, which had been treated with dGuo and further depleted of remaining CD45+ cells, and a mixture of two types of sorted CD4+ CD8+ (DP) thymocytes: monoclonal CD45.2 OT-II and polyclonal CD45.1 B6. OVAp recognized by the OT-II cells was added to the culture medium in serial dilutions as indicated. All of the cells recovered from each RTOC were run through and acquired for the FACS analysis (∼1–2.10⁵ cells/RTOC); therefore, the absolute numbers shown in every RTOC figure correspond to the total number of cells present in each RTOC. (A) Scheme of mixed OT-II/B6 RTOCs with OVAp in the medium. (B) Representative FACS plots for the OT-II CD4/CD8 profile (upper panels) and OT-II Treg development (lower panels) after 5 d of culture. The numbers on the FACS plots correspond to the absolute number of OT-II SP4 cells (upper panels) and Tregs (lower panels). (C) Proportion of SP8, DN, and SP4 cells within the OT-II cells in each condition. Error bars correspond to the SEM. (D) Percentages of Tregs in OT-II SP4 cells in each condition. Each symbol represents a single RTOC. Some conditions of this experiment were repeated more than two times.
We found that the epithelium alone can have dramatically different effects on SP4 and Treg development, depending on the amount of Ag present. Thus, in the absence of the cognate Ag or at the lowest doses tested, monoclonal OT-II thymocytes developed normally into SP4 cells. However, as the Ag concentration increased (as a surrogate for increased avidity), there was a reduction and then total elimination of OT-II SP4 cells, with a concomitant appearance of mature OT-II DN (51, 52) and SP8 cells. These mature OT-II DN and SP8 cells express a TCR complex with nonmatching TCR restriction and coreceptor, because OT-II TCR is MHCII restricted. Further, they may develop from existent SP4 cells that downregulate CD4 and mature into DN and even SP8 cells, or else develop independently. In the first case, the disappearance of the SP4 cells would be caused by divergence, whereas in the second case, it would be caused by actual deletion by the epithelium.

With regard to Treg development, there have been reports that Foxp3 is transiently expressed in activated non-Treg T cells in humans (53), but this does not seem to be the case in mice, in which the correlation between Foxp3 and Treg status appears to be consistent (54). The Foxp3+ CD25+ CD4 T cells generated in our cultures are assumed to be Tregs, although this could not be confirmed with functional tests because the number of Foxp3+ cells generated in our RTOCs was too low.

In the OT-II/B6 RTOCs with thymic epithelium alone, OT-II Tregs did not develop in the absence of the cognate Ag, but they appeared with intermediate doses. In agreement with published data (4), optimal TCR signals for Treg development in our cultures were stronger (assuming the Ag concentration is a surrogate for avidity) than for non-Treg SP4 development and partially overlapped with negative selection. This supports the notion of Tregs being resistant to deletion (4). Moreover, at the highest Ag doses tested, absolute numbers of Tregs and their proportion among SP4 cells decreased, indicating either increased deletion of Tregs or reduced commitment. Taken together, these results suggest an optimal avidity window for Treg development. Further, one may expect that, in conditions of highly efficient negative selection, as for strongly expressed Ags in the thymus, the production of Tregs with that specificity would be less critical.

In all RTOC experiments, the fetal thymic stroma (as the source of the thymic epithelium) had been carefully depleted of CD45+ cells, and the thymocytes used were sorted for DP cells to avoid in situ development of THSC from early precursors. Further, the effects observed when epithelial cells presented the cognate Ag to OT-II thymocytes were qualitatively different from those observed when THSCs were present, in terms of deletion, maturation of DN and SP8 cells, and Treg development. Thus, the observed results in experiments with epithelium alone are unlikely to be caused by contaminating hematopoietic cells. Overall, we showed that the thymic epithelium alone was able to eliminate autoreactive SP4 cells and support Treg development in an Ag dose–dependent manner. This is in agreement with previous reports (31, 39, 42).

To assess the role of THSC types in the establishment of self-tolerance, we supplemented RTOCs with highly purified individual THSC populations. Our system was designed to closely resemble a normal thymus, but with the advantage of having highly...
controlled cell type compositions, making it possible to unambiguously attribute the observed effects to one specific cell type.

In these cultures, Tregs and non-Treg SP4 cells were highly sensitive, with regard to deletion, to the THSC type added to the cultures in both a pure polyclonal and in a mixed polyclonal/monoclonal system. SP4 cells were strongly deleted in the presence of the two types of cDCs, as well as to a lesser extent in the presence of MΦs. No other THSCs tested showed a strong, reproducible ability for clonal deletion. Hematopoietic cells, in general, and DCs, in particular, have long been associated with CD4 tolerance (8, 13, 34).

Treg development was also highly dependent on the type of THSC that presented the Ag in the cultures. The two types of cDCs were markedly more efficient at supporting Treg development than any other THSC tested. Interestingly, cDCs were capable of both inducing Tregs and deleting them. In the polyclonal system, the presence of cDCs resulted in a reduced number of Tregs compared with the total absence of THSCs, indicating that they are able to negatively select Tregs. However, in the monoclonal system, cDCs presenting the cognate Ag induced Treg development, indicating that they are also able to support Treg maturation. Thus, the reduced number seen in the polyclonal system was likely the result of a combination of negative selection and induction of Tregs. Finally, we showed that Treg development supported by cDCthys was Ag specific and proportional to the Ag dose. These data are consistent with previous reports that associated cDCs with Treg development in the thymus (15, 18, 19).

Table I. Summary for the role of the different thymic stromal subsets in negative selection and Treg development

<table>
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<th>Thy Ep</th>
<th>pDC</th>
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<th>cDCthys</th>
<th>LB</th>
<th>MΦ</th>
<th>Eo</th>
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<td>Treg development</td>
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<td>+++</td>
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*Deletion or deviation to mature SP8 and DN.

The observed effects of the thymic epithelium and THSC types on developing thymocytes in RTOCs are summarized in this table. Note that the effect of the thymic epithelium cannot be compared to the effect of THSC types, as the experimental conditions are not equivalent.

Thy Ep, Thymic epithelium.
The two cDC populations showed similar results in our cultures. However, their roles in vivo are probably not redundant, because the Ags they come in contact with, and present to developing thymocytes, are different. While cDCthys sample the thymic milieu, cDCcers bring in Ag sampled from the periphery (21).

In addition to the controls already discussed, such as the use of DP thymocytes and the careful depletion of CD45R0 cells from the thymic stroma, other aspects were considered. For example, by using a short peptide (OVAp) we avoided the bias introduced by the chosen model Ag, in terms of abundance and localization (membrane bound or soluble), and the different capacity of thymic APCs to sample the environment (21). Moreover, transfer of Ag should not alter the interpretation of our results because it was shown to be unidirectional from mTECs to DCs (55), and whenever these two populations were together in an RTOC, only the DCs presented the Ag. Furthermore, the use of a cognate peptide to stimulate the generation of Tregs overcomes the finite number of niches that are available when using endogenous peptides and prevents the intraclonal competition that was observed previously (50). Finally, the use of RTOCs supplemented with purified THSC populations allowed us to overcome the difficulty of assigning the observed effects to a single APC population in complex in vivo systems (46).

In summary, we characterized and quantified six populations of THSCs present in the adult thymus and assessed their capacity to induce CD4 tolerance to self in a comparative analysis in parallel with the thymic epithelium. We showed that the thymic epithelium alone is capable of promoting self-tolerance by eliminating autoreactive SP4 thymocytes and supporting Treg development. Further, among THSCs, the two populations of cDCs, and MΦs to a lower extent, efficiently deleted both non-Treg SP4 cells and Tregs. The same two populations of cDCs were also the most efficient THSCs at supporting Treg development. The latter result shows that, although cDCs and MΦs may share some characteristics required for negative selection of autoreactive SP4 cells, they do not share those required for supporting Treg development.

This highlights a qualitative functional difference between these two populations and makes cDCs a functionally nonredundant cell type. Further, from the T cell point of view, this suggests a qualitative difference in the requirements for negative selection and Treg induction, which may operate together with the quantitative requirements in terms of TCR signal strength (4).

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Disclosures

The authors have no financial conflicts of interest.

References


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SUPPLEMENTAL FIGURE 1

SUPPLEMENTAL FIGURE 1. Characterization of the splenic counterparts of THSC.
Total digests of adult B6 spleens were enriched for cells expressing CD11c or CD19, using magnetic beads. The enriched populations were subsequently stained with the relevant antibodies for FACS analysis. Here, the expression of a variety of markers on THSC (black line) and their splenic counterparts (red line) is shown. The background fluorescence was determined by omitting only the relevant antibody (Full minus one, FMO) (solid grey for THSC and solid red for the splenic counterparts). pDC = plasmacytoid dendritic cell; cDCrec = recirculating conventional DC; cDCthy = intrathymically developed cDC; LB = lymphocyte B; M = macrophage and Eo = eosinophil.
SUPPLEMENTAL FIGURE 2. Depletion of remaining macrophages from the dGuo-treated fetal thymic stroma, gating strategy for the analysis of B6/OTII RTOCs and thymocyte maturation after 5 days of culture.

(A) RTOCs were constituted of immature thymocytes and fetal thymic stroma that had been treated with dGuo and further depleted of remaining CD45+ cells with magnetic beads to completely eliminate hematopoietic cells. Representative FACS plots for the dGuo-treated fetal thymic stroma before and after depletion of CD45+ cells are shown (left panels). Representative FACS plots for the positive fraction of the CD45 depletion are shown (right panels). (B) Mixed monoclonal/polyclonal RTOCs were constituted of fetal thymic stroma depleted from hematopoietic cells, and a mixture of two types of sorted CD4+CD8+ DP thymocytes: monoclonal CD45.2 OTII and polyclonal CD45.1 B6. The cognate antigen (OVAp) was either added to the medium or preloaded on the supplemented THSC as indicated on each experiment. After 5 days of culture, RTOCs were digested, stained with the relevant antibodies and analyzed by FACS. Here, the gating strategy for the analysis of every RTOC is shown on representative FACS dot plots from RTOCs supplemented with loaded-THSCs. Note that after the 5 days of culture all the sorted immature DP thymocytes in the RTOCs have matured into SP. This was the case for every experiment in this paper.
SUPPLEMENTAL FIGURE 3. Eo-dependent reduced number of thymocytes is not antigen-specific but due to experimental handling.

Polyclonal B6 RTOCs performed as described in Figure 3 were supplemented as indicated with Eo that had been subjected to a mock-OVAp loading treatment (incubation and extensive washing without the actual presence of OVAp) (Mock OVAp-loaded Eo) or untreated (Unt Eo). Here, the normalization of Non-Treg SP4 (grey bars) and Treg (white bars) numbers is shown. Error bars correspond to the standard error of the mean (SEM). Asterisks indicate the statistical significance of differences between each condition and non-supplemented RTOCs using Mann-Whitney non-parametric test. * = p<0.05; ** = p<0.01; *** = p<0.001.
SUPPLEMENTAL FIGURE 4

(A) Similar effect of THSC types on the development of B6 thymocytes in both pure B6 and in mixed OT-II/B6 RTOCs, and specificity of the observed effect.

(B) Specificity of the observed effect with cDCth. Absolute numbers of Non-Treg SP4 in OTII thymocytes after 5 days of culture, from the same type of RTOCs of Fig. 5. Here, the supplementing cDCth had not been previously loaded with OVAp, so they did not present the cognate Ag to OT-II thymocytes. Error bars correspond to the standard error of the mean (SEM). Asterisks indicate the statistical significance of differences between each condition and non-supplemented RTOCs using Mann-Whitney non-parametric test. * = p<0.05; ** = p<0.01; *** = p<0.001.