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# IL-2 prevents deletion of developing T-regulatory cells in the thymus

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**In the thymus, strongly self-reactive T cells may undergo apoptotic deletion or differentiate into Foxp3+ T-regulatory (T-reg) cells. Mechanisms that partition T cells into these two fates are unclear. Here, we show that IL-2 signalling is required to prevent deletion of CD4+ CD8– CCR7+ Helios+ thymocytes poised to upregulate Foxp3. The deletion prevented by IL-2 signalling is Foxp3 independent and occurs later in thymocyte development than the deletion that is prevented by Card11 signalling. Our results distinguish two bottlenecks at which strongly self-reactive thymocytes undergo deletion or progress to the next stage of T-reg differentiation; Card11 regulates the first bottleneck and IL-2 regulates the second.**
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During T-cell development in the thymus, TCR signalling strength plays a critical role in determining T-cell fate. While weak TCR signalling induces thymocytes to develop into naïve T cells, strong TCR signalling induces alternative fates, including apoptotic deletion or Foxp3+ T-reg differentiation. Whereas deletion can occur at any stage of thymocyte development,[1–3] upregulation of Foxp3 during T-reg differentiation occurs mainly in mature CD4+ CD8– (CD4 single positive, CD4SP) thymocytes that express the chemokine receptor, CCR7.[4,5] The strongly TCR signalled CD4SP CCR7+ thymocyte population thus contains cells that are poised for deletion or poised for Foxp3+ T-reg differentiation (i.e. T-reg precursor), but the signals and steps that determine this cell fate decision are only partially understood.

Helios is the only molecular marker known to be upregulated by strong TCR signalling and downregulated by weak TCR signalling in thymocytes.[6] Mice with defective apoptosis have an increased number of CD4SP CCR7+ Helios+ Foxp3– cells and Foxp3+ T-reg cells in the thymus, while these populations are diminished in mice that lack Card11 (also called CARMA1) or c-Rel.[6–10] Card11-deficient mice with defective apoptosis have a substantial CD4SP CCR7+ Helios+ Foxp3– thymocyte population[6] but Foxp3+ cells are still absent.[9] These data reveal two essential and distinct functions of Card11 in T-reg differentiation: to prevent apoptotic deletion of T-reg precursors and to mediate Foxp3 upregulation in T-reg precursors.

Thymic T-reg differentiation has been characterised as a two-step process consisting of strong TCR signalling followed by cytokine-induced Foxp3 upregulation.[11] IL-2 and IL-15 are members of a cytokine family that signals via the cytokine receptor, CD132 (the common $\gamma$-chain).[12] Mice lacking CD132, or the $\alpha$ or $\beta$ subunits of the IL-2 receptor, have very few Foxp3+ T-reg cells in the thymus.[13,14] However, CD132-deficient mice with a defective apoptosis pathway have a substantial population of thymic Foxp3+ T-reg cells.[8] It has been postulated that cytokine signalling is required to prevent deletion induced by strong TCR signalling.[15] A competing hypothesis proposes that cytokine signalling is required to counteract a proapoptotic protein signature, which is induced in developing T-reg cells by Foxp3.[8] Distinguishing between these possibilities is essential to advance our understanding of how thymocytes partition into deletion *versus* T-reg differentiation fates.

To address this, we examined the stage(s) at which various genetic defects impinge on the development of strongly TCR signalled thymocytes. We found that IL-2 signalling is essential to prevent deletion of CD4SP CCR7+ Helios+ thymocytes at a later developmental stage than Card11 is required to prevent deletion. The deletion prevented by IL-2 signalling occurs in a Foxp3-independent manner. We propose that variation in Card11 and IL-2 signalling determines whether CD4SP CCR7+ thymocytes undergo deletion or progress to the next stage of Foxp3+ T-reg differentiation.

## Results

**CD4SP thymocytes able to respond to IL-2 express CCR7 and Helios.** To test whether CD4SP thymocytes at distinct developmental stages are differentially responsive to IL-2, thymocytes from *Foxp3$^{GFP}$* mice were sorted into three subsets of CD4SP Foxp3– cells: the least mature CCR7– CD24+ cells, semi-mature CCR7+ CD24+ cells and most mature CCR7+ CD24– cells[6] (Figure 1a). This sorting strategy was used, in part, to exclude NKT cells and non-nascent T-reg precursor cells, which have a CCR7– CD24– phenotype (Figure 1b and ref. 16). After 20 h, the frequency of lymphocytes among ungated events was significantly lower in CCR7– CD24+ cultures compared with the other

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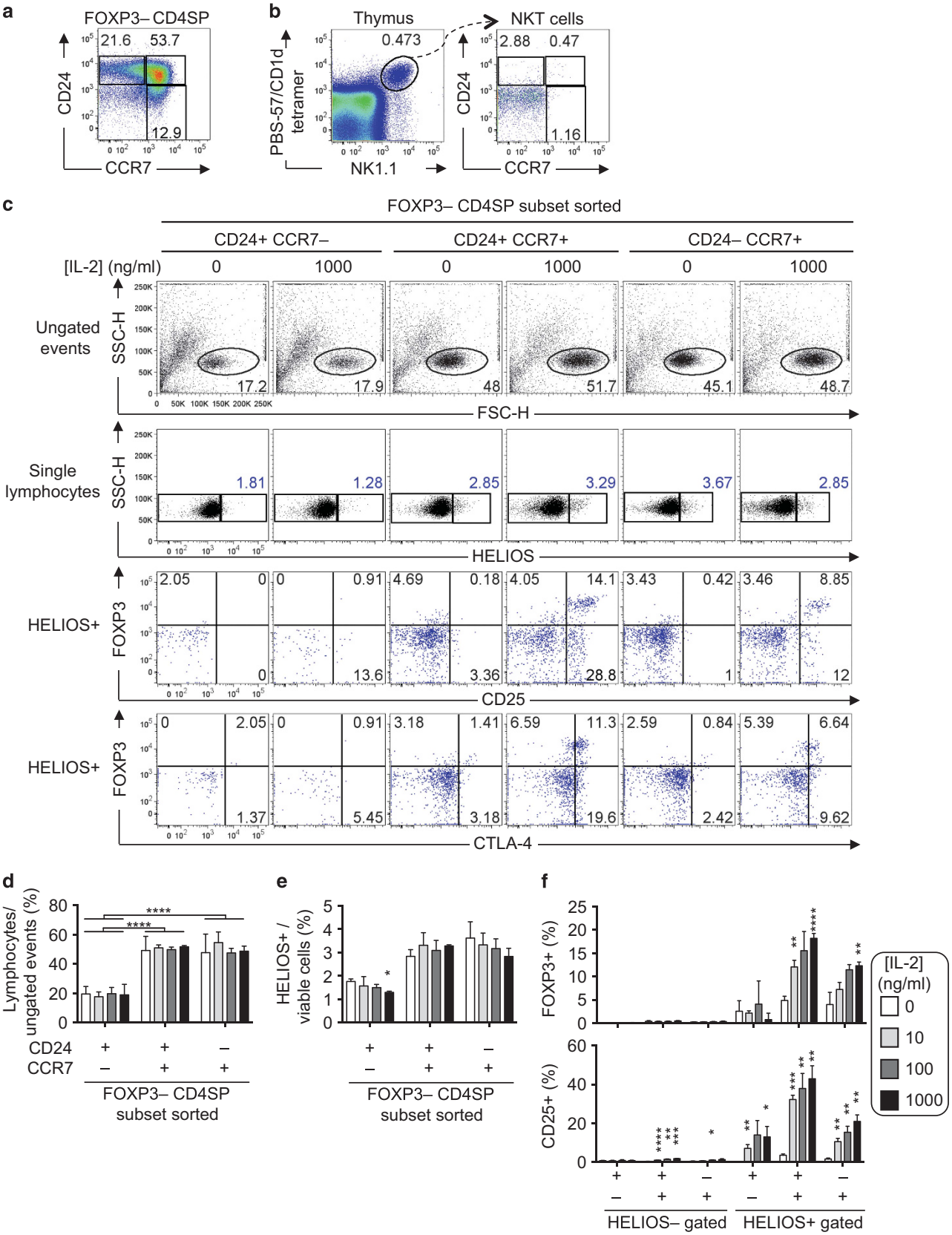
a
FOXP3– CD4SP
21.6
53.7
12.9
CD24
CCR7
b
Thymus
0.473
PBS-57/CD1d tetramer
NK1.1
NKT cells
2.88
0.47
1.16
CD24
CCR7
c
FOXP3– CD4SP subset sorted
CD24+ CCR7–
CD24+ CCR7+
CD24– CCR7+
[IL-2] (ng/ml)
0
1000
0
1000
0
1000
Ungated events
SSC-H
FSC-H
17.2
17.9
48
51.7
45.1
48.7
Single lymphocytes
HELIOS
1.81
1.28
2.85
3.29
3.67
2.85
HELIOS+
FOXP3
CD25
2.05
0
0
0.91
4.69
0.18
4.05
14.1
3.43
0.42
3.46
8.85
0
13.6
3.36
28.8
1
12
HELIOS+
FOXP3
CTLA-4
0
2.05
0
0.91
3.18
1.41
6.59
11.3
2.59
0.84
5.39
6.64
1.37
5.45
3.18
19.6
2.42
9.62
d
Lymphocytes/ ungated events (%)
****
****
CD24
CCR7
FOXP3– CD4SP subset sorted
e
HELIOS+ / viable cells (%)
FOXP3– CD4SP subset sorted
f
FOXP3+ (%)
CD25+ (%)
[IL-2] (ng/ml)
0
10
100
1000
HELIOS– gated
HELIOS+ gated

subsets (Figures 1c, top row and d), consistent with reduced survival of CCR7– CD24+ cells. The addition of IL-2 to the cultures had no significant effect on the frequency of lymphocytes detected, nor the frequency of Helios+ cells, at any maturation stage (Figures 1c, second row and d and e). These data provided no evidence that IL-2 induced preferential survival of Helios– or Helios+ thymocytes, nor was there evidence that IL-2 induced Helios expression, during these short-term cultures. The Foxp3+ cell frequency among CCR7+ Helios+ cells was significantly higher in the presence of 1000 ng/ml IL-2 than in cultures with no IL-2 added, but this was not observed in Helios+ cells at the least mature CCR7– CD24+ stage, nor in Helios– cells at any maturation stage (Figures 1c and f). Most Foxp3+ cells expressed CD25, but Foxp3+ CD25– and Foxp3– CD25+ populations were also detectable, with the highest CD25+ cell frequencies observed among CCR7+ Helios+ cells (Figures 1c and f). CTLA-4 was also detectable in a subset of the Foxp3+ cells (Figure 1c). These results show that the CCR7+ Helios+ subset of CD4SP thymocytes is enriched in cells capable of responding to IL-2 by expressing one or more of the T-reg-associated proteins, Foxp3, CD25 and CTLA-4.

**Foxp3 is not required for CTLA-4 induction in CD4SP CCR7+ Helios+ thymocytes.** Among CD4SP CCR7+ thymocytes, we noted that the Helios+ Foxp3– subset expressed less CTLA-4 than the Foxp3+ subset (Figure 2a). As CTLA-4 and Foxp3 expression were correlated, and Foxp3 binds to the *Ctla4* gene,[17] we tested whether Foxp3 is required for CTLA-4 expression by examining mice bearing a *Foxp3*$^{null}$ allele.[18] Normal frequencies of CTLA-4+ cells were detected among CD4SP CCR7+ thymocytes from *Foxp3*$^{+/null}$ and *Foxp3*$^{null/y}$ mice (Figures 2b and c). Approximately 80% of CD4SP CCR7+ CTLA-4+ thymocytes were Helios+, independent of *Foxp3* genotype (Figures 2b and d). Thus, CTLA-4 upregulation occurs independently of Foxp3 in CD4SP CCR7+ thymocytes, predominantly in cells that express high levels of Helios.

**IL-2 is required for Foxp3 and CTLA-4 expression in CD4SP CCR7+ Helios+ thymocytes.** To investigate the effects of IL-2 on strongly TCR signalled thymocytes *in vivo*, we first compared CD4SP CCR7+ thymocytes in IL-2-sufficient and IL-2-deficient mice. To avoid potential effects of systemic immune dysregulation in *Il2*$^{-/-}$ mice,[19] we examined the pups from three litters at 14–15 days after birth, before the onset of overt disease. The Foxp3+ T-reg cell frequency among CD4SP CCR7+ thymocytes was significantly decreased in *Il2*$^{-/-}$ mice (Figure 3a). In contrast, there were no significant differences in Helios+ Foxp3– cell frequencies within CD4SP CCR7+ thymocytes (Figure 3a).

To examine the consequences of cell-autonomous deficiency in IL-2 signalling, we used mice with an inactivating mutation in the *Il2ra* gene, which encodes CD25. Irradiated mice were reconstituted with wild-type bone marrow (BM) mixed with *Il2ra*$^{+/+}$, *Il2ra*$^{+/-}$ or *Il2ra*$^{-/-}$ BM. At 10 weeks after BM reconstitution, there was a significant decrease in the Foxp3+ T-reg cell frequency among CD4SP CCR7+ thymocytes derived from *Il2ra*$^{+/-}$ or *Il2ra*$^{-/-}$ BM (Figure 3b). However, there were no significant differences in Helios+ Foxp3– cells (Figure 3b).

As IL-2 was sufficient to induce CTLA-4 *in vitro* (Figure 1), we examined whether IL-2 is necessary for CTLA-4 induction *in vivo*. CTLA-4 expression on Foxp3+ cells was decreased in a manner dependent on the *Il2* or *Il2ra* gene dose (Figures 3a and b). Although dispensable for the generation of CD4SP CCR7+ Helios+ Foxp3– thymocytes, IL-2 signalling is sensitively required for the transition to the next stage of T-reg differentiation, which is characterised by upregulation of Foxp3 and/or CTLA-4.

**IL-15 can partially substitute for IL-2 in thymic Foxp3+ T-reg differentiation.** To test for effects of IL-15 on strongly TCR signalled CD4SP CCR7+ thymocytes, we examined *Il15*$^{-/-}$ mice. By most parameters *Il15*$^{-/-}$ mice were indistinguishable from wild-type littermate controls, except *Il15*$^{-/-}$ mice had a slightly lower frequency, but not number, of Helios+ Foxp3– thymocytes in the CCR7+ CD4SP population (Figure 4a). *Il2*$^{-/-}$ *Il15*$^{-/-}$ double-deficient mice had a significantly lower frequency of Foxp3+ cells than did *Il2*$^{-/-}$ mice (Figure 4a). However, *Il2*$^{-/-}$ *Il15*$^{-/-}$ mice had a significant increase in the number of Helios+ Foxp3– cells compared with all other groups (Figure 4a), indicating that the defect could not be attributed to loss of T-reg precursors. CTLA-4 expression was low to undetectable on thymic Foxp3+ T-reg cells from *Il2*$^{-/-}$ and *Il2*$^{-/-}$ *Il15*$^{-/-}$ mice, significantly lower than the *Il15*$^{-/-}$ group (Figure 4a, bottom row). These findings are consistent with the hypothesis that IL-15 contributes to thymic T-reg development when IL-2 is absent.[20] They also indicate

**Figure 1** The CCR7+ Helios+ subset of CD4SP thymocytes is enriched in cells that can respond to IL-2. (**a**) CD24/CCR7 phenotype of Foxp3– CD4SP thymocytes from B6 mice showing gating for CD24– CCR7+, CD24+ CCR7+ and CD24– CCR7+ subsets. (**b**) CD24– CCR7– phenotype of thymic NKT cells. Left plot shows total thymocytes from a B6 mouse stained with anti-NK1.1 plus a tetramer of mouse CD1d loaded with the $\alpha$-galactosylceramide analogue, PBS-57. Thymic NKT (PBS-57/CD1d+ NK1.1+) cells are analysed for CD24/CCR7 phenotype in the right plot. Results are representative of two experiments. (**c**) Foxp3-GFP– CD4SP thymocytes were FACS sorted into three subsets based on CCR7 and CD24 expression and cultured for 20 h in the presence or absence of IL-2 (denoted above plots). Top row shows side-scattered light amplitude (SSC-H) *versus* forward-scattered light amplitude (FSC-H) for all events in the flow cytometry datafiles and the gate used to define lymphocytes. After excluding doublets based on FSC width and SSC width (gates not shown), single lymphocytes were analysed for Helios expression in the second row. Gated Helios+ events were analysed for Foxp3/CD25 (third row) and Foxp3/CTLA-4 (fourth row) phenotypes. (**d**) Lymphocyte frequency among ungated events as shown in top row of (**c**). (**e**) Helios+ cell frequency among single lymphocytes as shown in second row of (**c**). (**f**) Foxp3+ (top) or CD25+ (bottom) cell frequency among Helios– or Helios+ cells after 20 h in culture in the presence or absence of graded concentrations of IL-2 as gated in third and fourth rows of (**c**). Columns and error bars represent mean and s.e.m. of data obtained from three mice. Similar data were obtained in a repeat experiment. Statistical analyses used were (**d**) two-way ANOVA with Sidak's post-test or (**e** and **f**) multiple *t*-tests comparing each concentration of exogenous IL-2 to the 0 ng/ml IL-2 condition for each T-cell subset, without assuming a consistent standard deviation, with Holm–Sidak post-tests. Multiplicity adjusted *P*-value symbols: *

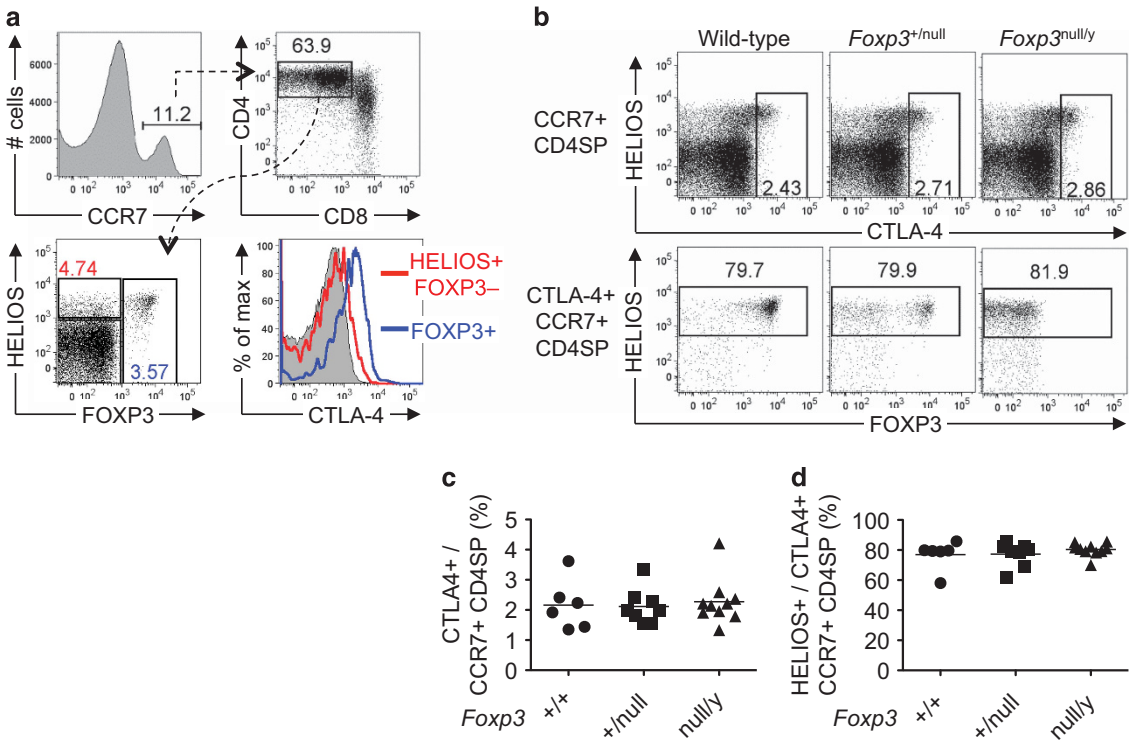


**Figure 2** CTLA-4 upregulation in CD4SP CCR7+ Helios+ cells is Foxp3 independent. (**a**) Gating strategy used to distinguish three subsets within the CCR7+ CD4SP thymocyte population based on Helios/Foxp3 phenotype, and CTLA-4 expression on these three subsets. (**b**) Helios/CTLA-4 phenotype (upper panel) and Helios/Foxp3 phenotype on the CTLA-4+ subset (lower panel) of CCR7+ CD4SP thymocytes from B6 mice ('Wild-type') or female B6.*Foxp3*$^{+/null}$ and male B6.*Foxp3*$^{null/y}$ littermates aged 6–20 days. (**c**, **d**) Graphs show the frequencies of (**c**) CTLA-4+ cells or (**d**) Helios+ cells, as gated in (**b**), with each symbol representing one mouse

a requirement for IL-2, but not IL-15, in maintaining CTLA-4 expression on thymic T-reg cells.

To examine the role of cell-autonomous IL-2 and IL-15 signalling in a context free of systemic immune dysregulation, mixed chimeras were generated in which some haemopoietic cells lacked expression of CD122 (encoded by *Il2rb*), a receptor for IL-2 and IL-15.[21] At 8 weeks after reconstitution, no change was observed in the Helios+ Foxp3– cell frequency derived from *Il2rb*$^{-/-}$ BM, but there was a significant decrease in the Foxp3+ T-reg cell frequency derived from *Il2rb*$^{-/-}$ BM (Figure 4b). CTLA-4 expression on T-reg cells was also reduced in cells derived from *Il2rb*$^{-/-}$ BM (Figure 4b). Thus, a similar phenotype was observed in CD4SP CCR7+ thymocytes lacking either CD25 or CD122, with no detectable defect in the Helios+ Foxp3– population, but a sharp decrease in Foxp3+ T-reg cells.

**The onset of IL-2 dependence coincides with Foxp3 upregulation.** While the results above suggest that the onset of IL-2 dependence and Foxp3 upregulation coincide in CD4SP CCR7+ Helios+ thymocytes, some of the cells detected may have derived from mature cells that entered the thymus from the periphery.[16,22] To focus our analysis on a synchronised cohort of nascent thymocytes, we injected mice with 5-ethynyl-2'-deoxyuridine (EdU), which is incorporated into DNA-synthesising thymocytes predominantly at the stage immediately preceding $\alpha\beta$ TCR-dependent selection.[5] In a time-course experiment we demonstrated that nascent EdU+ T-reg cells peak at 6-8 days after EdU injection.[5] Seven days after the mixed chimeras described in Figure 4b were injected with EdU, the EdU+ CD4SP CCR7+ population derived from *Il2rb*$^{-/-}$ BM cells showed a selective deficiency of Foxp3+ cells, but Helios+ Foxp3– cells were present at normal frequencies (Figure 4c). This indicates that IL-2 signalling is required for the transition from the Helios+ Foxp3– stage to the Foxp3+ T-reg stage of thymic T-reg differentiation.

**Foxp3 is not required for thymocyte deletion.** Since the CD25-deficient and CD122-deficient cells studied above had normal *Foxp3* genes, it remained possible that Foxp3 was responsible for deleting the thymocytes that could not respond to IL-2 or IL-15, as recently hypothesised.[8] To test this possibility we mixed BM from wild-type CD45.1+ mice and CD45.2+ donor mice with inactivating mutations in the *Il2rb* and/or *Foxp3* genes and generated mixed chimeras as above. The chimeric mice were injected with EdU 7 days before analysis of EdU+ CD4SP CCR7+ CD45.2+ thymocytes. Foxp3-deficiency alone had no effect on the frequency of Helios+ cells, whereas CD122 deficiency significantly decreased the frequency of Helios+ cells (Figure 5b). If CD122 signalling were required to counteract Foxp3-dependent deletion, it would be hypothesised that the Helios+ cell frequency would increase in Foxp3/CD122 double-deficient cells in comparison with CD122 single-deficient cells. This was not the case, as similar frequencies

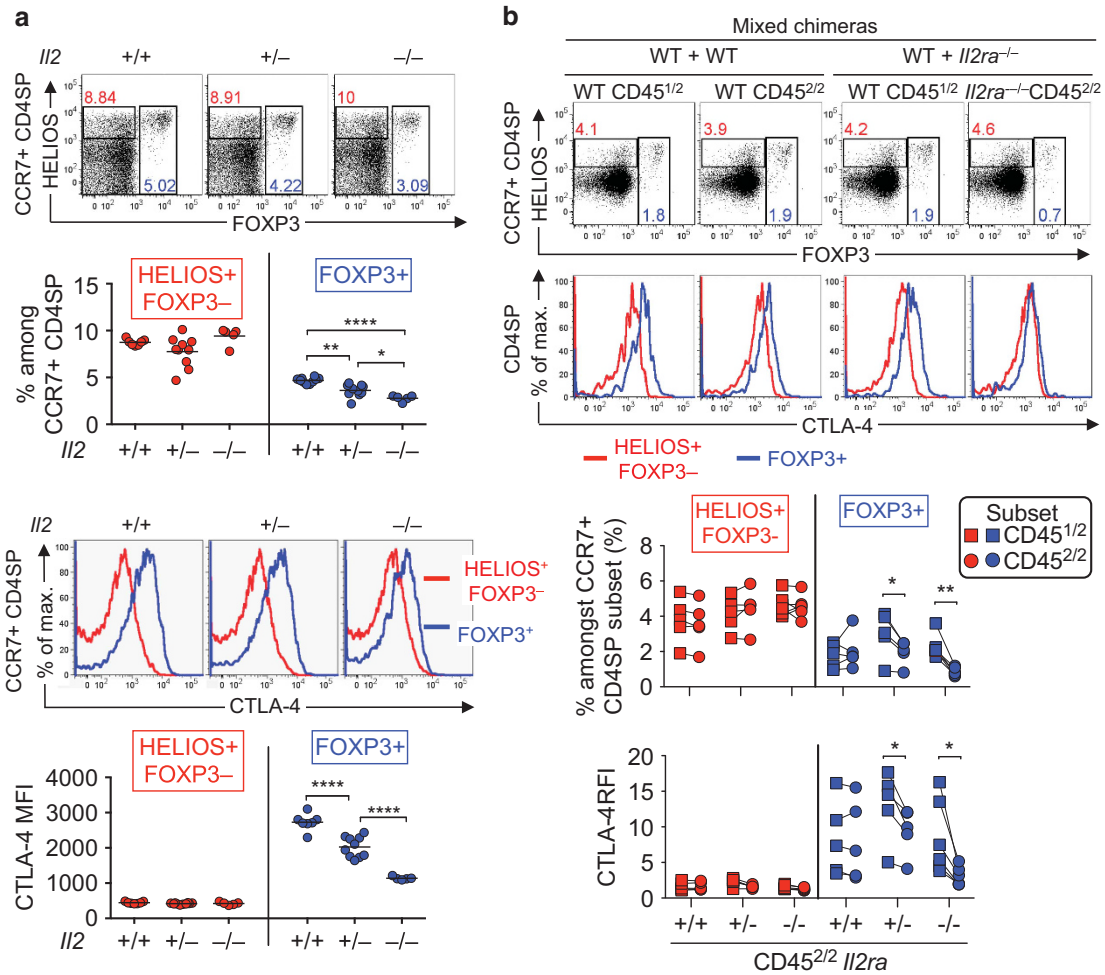


**Figure 3** Foxp3 and CTLA-4 upregulation in CD4SP CCR7+ Helios+ cells is IL-2 dependent. (**a**) Upper plots show Helios/Foxp3 phenotype of CCR7+ CD4SP thymocytes from *Il2*$^{+/+}$, *Il2*$^{+/-}$ and *Il2*$^{-/-}$ littermates on the B10.BR background examined at 14–15 days after birth. Lower histograms show CTLA-4 expression on Helios+ Foxp3– (red) and Foxp3+ (blue) subsets of CCR7+ CD4SP thymocytes from the same mice. Summaries (below) show the frequencies, or CTLA-4 MFI, of Helios+ Foxp3– or Foxp3+ cells as gated in the plots, with each symbol representing one mouse. (**b**) Helios/Foxp3 phenotype of CD45$^{1/2}$ and CD45$^{2/2}$ subsets of CCR7+ CD4SP thymocytes (top row), and CTLA-4 expression on their Helios+ Foxp3– and Foxp3+ subsets (bottom row), from CD45$^{1/1}$ mice that were irradiated and reconstituted 6–10 weeks earlier with BM mixtures from CD45$^{1/2}$ wild-type mice plus CD45$^{2/2}$ mice that were either *Il2ra*$^{+/+}$, *Il2ra*$^{+/-}$ or *Il2ra*$^{-/-}$. Graphs show the frequencies of populations gated in plots (top row) or the CTLA-4 relative fluorescence intensity (RFI) (bottom row, normalised to the CCR7+ CD4SP Helios– Foxp3– subset in the same sample). Lines join measurements from an individual mouse. Data in (**a**) and (**b**) were compiled from two and three experiments, respectively. Two-tailed Student's *t*-tests (unpaired in **a**; paired in **b**) *P*-value symbols: * 0.01–0.05, ** 0.001–0.01, **** $<0.0001$

of Helios+ cells were observed (Figure 5b), indicating that Foxp3 is not required for deletion of CD4SP CCR7+ Helios+ thymocytes that lack CD122 expression. CD122 deficiency significantly decreased CTLA-4 expression on EdU+ CD4SP CCR7+ Helios+ thymocytes, whereas Foxp3-deficiency had no effect on CTLA-4 expression (Figure 5b). These results show that CD122 prevents a deletion mechanism that is Foxp3 independent.

**IL-2 signalling prevents deletion of developing T-reg cells at a later stage than Card11.** In contrast to the results above, CD4SP CCR7+ Helios+ Foxp3– thymocytes are decreased by loss-of-function mutations in *Card11* or *Rel*,[6] suggesting that TCR-CD28 signalling through Card11 to activate NF-κB/c-Rel is required at an earlier stage of thymic T-reg differentiation than IL-2. We wished to test that hypothesis directly, and simultaneously verify that many thymocytes with the potential to become T-reg cells undergo deletion mediated by the proapoptotic protein, Bim (encoded by the *Bcl2l11* gene). We irradiated B6.*Rag1*$^{-/-}$ mice and injected wild-type, *Bcl2l11*$^{-/-}$, *Card11*$^{unm/unm}$ or *Il2rb*$^{-/-}$ BM to reconstitute haematopoiesis. These chimeras were examined 5 days after EdU injection, which is when EdU+ CCR7+ Helios+ Foxp3– thymocytes peak and EdU+ CCR7+ Foxp3+ thymocytes are first detectable in substantial numbers.[5] Among EdU+ CD4SP CCR7+ cells, the frequency of Helios+ Foxp3– cells was increased in recipients of *Bcl2l11*$^{-/-}$ BM and decreased in recipients of *Card11*$^{unm/unm}$ BM (Figure 6), as previously observed in non-chimeric mice.[6] In contrast, the Helios+ Foxp3– population in recipients of *Il2rb*$^{-/-}$ BM was not significantly different from wild-type controls (Figure 6). The frequency of Foxp3+ cells was increased in

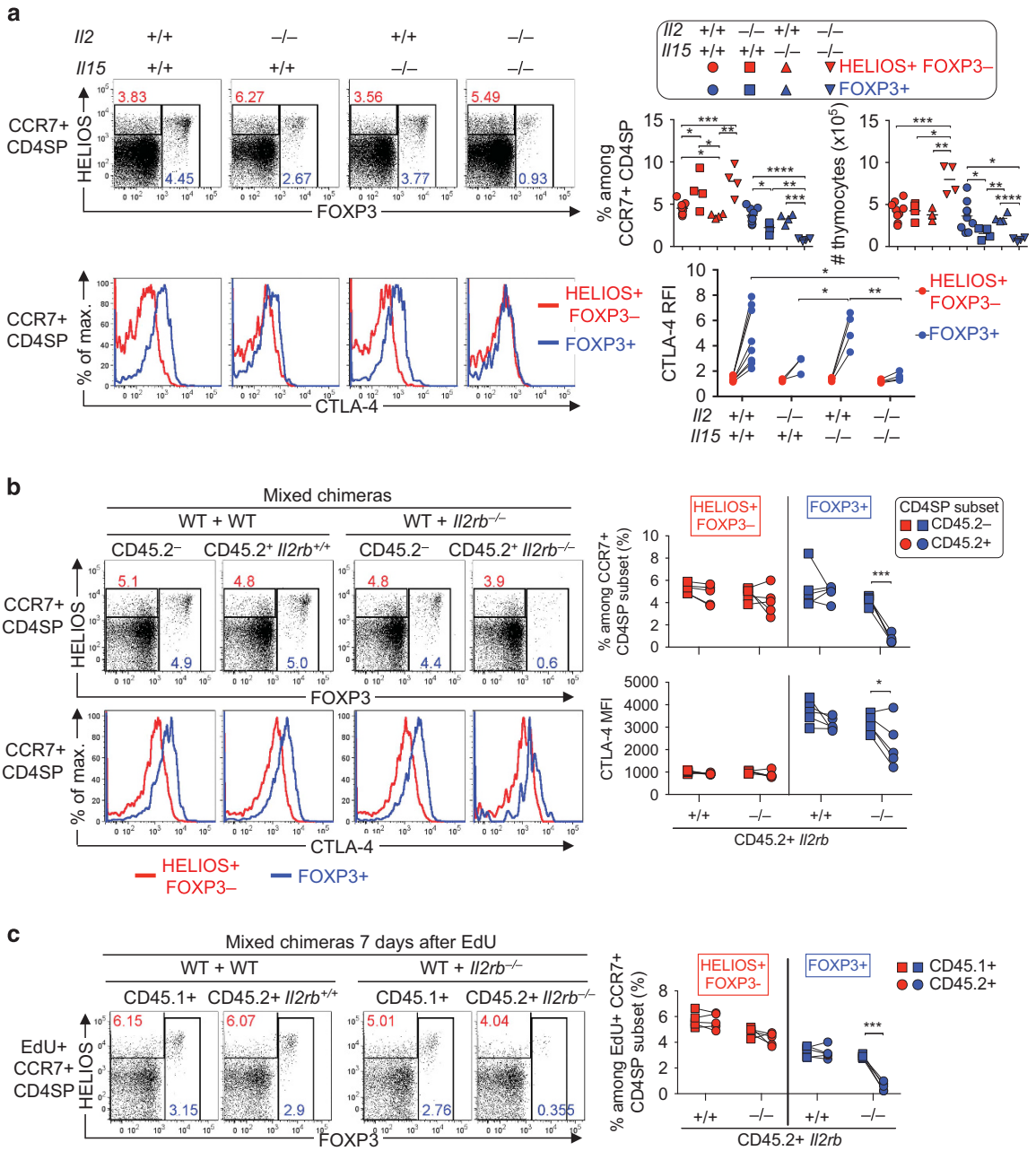


**Figure 4** CD4SP CCR7+ Helios+ thymocytes that cannot respond to IL-2 or IL-15 are deleted. (**a**) Helios/Foxp3 phenotype of CCR7+ CD4SP thymocytes (top row) and CTLA-4 labelling on their Helios+ Foxp3– and Foxp3+ subsets (bottom) from wild type, *Il2*$^{-/-}$, *Il15*$^{-/-}$ and *Il2*$^{-/-}$*Il15*$^{-/-}$ mice on the B10.BR background examined at 28-47 days after birth. Graphs (right) show the frequency and absolute number of thymocyte subsets gated in plots (top row) or the CTLA-4 RFI (bottom row, normalised to the CCR7+ CD4SP Helios– Foxp3– subset in the same sample). (**b**) Helios/Foxp3 phenotype of CD45.2– and CD45.2+ subsets of CCR7+ CD4SP thymocytes (top row), and CTLA-4 labelling on their Helios+ Foxp3– and Foxp3+ subpopulations (bottom row), from B6.*Rag1*$^{-/-}$ mice that were irradiated and reconstituted 8 weeks earlier with BM mixtures from CD45.1+ wild-type mice plus CD45.2+ mice that were either *Il2rb*$^{+/+}$ or *Il2rb*$^{-/-}$. Graphs show the frequencies of populations gated in plots (top row) or the CTLA-4 MFI (bottom row). (**c**) Chimeric mice described in (**b**) were injected with EdU 7 days before analysis and EdU+ thymocytes were examined as in (**b**). Graph shows the frequencies of populations gated in plots. Lines on graphs join measurements from an individual mouse. Data in (**a**) were compiled from four experiments, (**b** and **c**) from one experiment. Two-tailed Student's *t*-tests (unpaired in **a** and paired in **b** and **c**) *P*-value symbols: * 0.01–0.05, ** 0.001–0.01, *** 0.0001–0.001; **** $<0.0001$

recipients of *Bcl2l11*$^{-/-}$ BM, and decreased in recipients of *Card11*$^{unm/unm}$ or *Il2rb*$^{-/-}$ BM, relative to wild-type controls (Figure 6). Thus, Card11 and IL-2 signals prevent Bim-dependent deletion at distinct stages of thymic T-reg cell differentiation, with Card11 required at the Helios+ Foxp3– stage, whereas IL-2 signalling is required later, approximately at the onset of Foxp3 expression.

## Discussion

The expression of Helios at high levels marks T cells bound for deletion at any stage of thymocyte development and also marks CD4SP CCR7+ thymocytes poised to upregulate Foxp3. To elucidate mechanisms that distinguish deletion and T-reg differentiation, we concentrated on CD4SP CCR7+

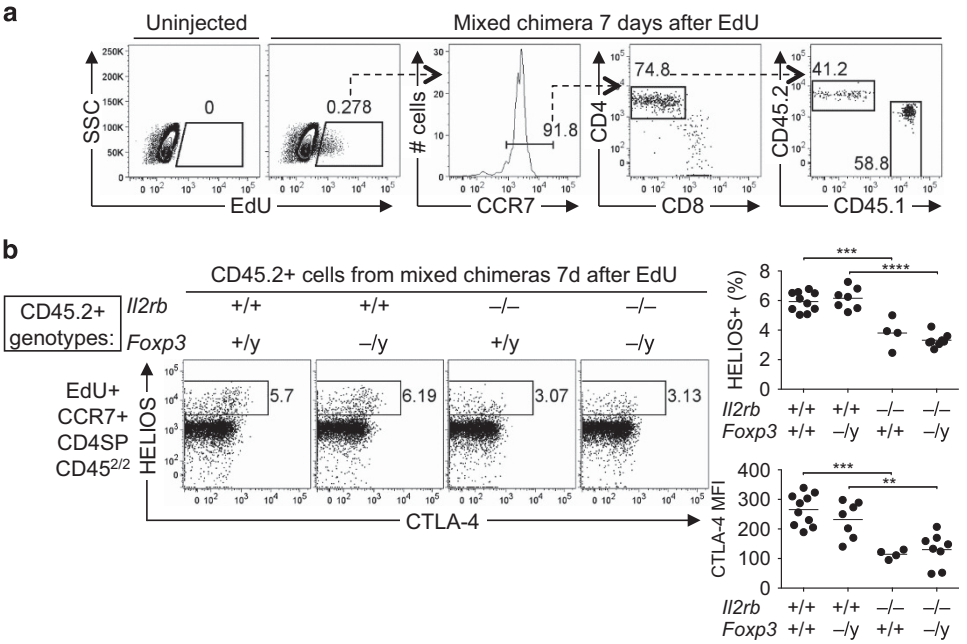


**Figure 5** Deletion of CD122-deficient CD4SP CCR7+ Helios+ cells is Foxp3 independent. CD45.1+ mice were irradiated and reconstituted with cell mixtures containing CD45.1+ wild-type BM plus CD45.2+ BM from wild-type, *Foxp3*$^{null/y}$, *Il2rb*$^{-/-}$ or *Il2rb*$^{-/-}$ *Foxp3*$^{null/y}$ siblings. Chimeric mice were examined 6–7 weeks later, 7 days after a single EdU injection. (**a**) Gating strategy to analyse nascent cells derived from thymocytes that were proliferating 7 days prior to analysis. Left plots show side-scattered light (SSC) *versus* EdU on all thymocytes, with a gate for EdU+ events that was empty in the uninjected control. The CCR7+ subset of EdU+ cells was analysed for CD4/CD8, and the CD4SP subset was then analysed for expression of CD45.1 *versus* CD45.2. (**b**) Plots show Helios/CTLA-4 phenotype of EdU+ CCR7+ CD4SP CD45.2+ cells. Graphs show the frequencies of Helios+ events (top) or the CTLA-4 MFI of Helios+ events (bottom) as gated in the plots. We generated nine chimeras per group, except for the group bearing wild-type BM cells, which contained 10 mice. Possibly due to intraperitoneal injection failure, EdU+ cells were not detected in thymus samples from some EdU-injected mice; these samples were excluded from the analysis. Graphs show data compiled from two independent experiments with each symbol representing one mouse. Unpaired two-tailed Student's *t*-tests *P*-value symbols: ** 0.001–0.01, *** 0.0001–0.001; **** $<0.0001$

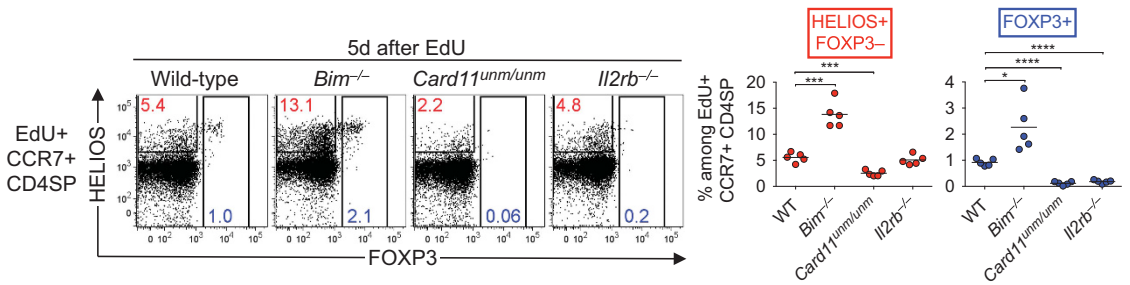


**Figure 6** CD122 prevents Bim-dependent deletion at a later stage than Card11. Helios/Foxp3 phenotypes of EdU+ CCR7+ CD4SP thymocytes 5 days after EdU injection of B6.*Rag1*$^{-/-}$ mice that were irradiated and reconstituted 35 days before analysis with BM from wild-type, *Bim*$^{-/-}$, *Card11*$^{unm/unm}$ or *Il2rb*$^{-/-}$ mice. Graphs (right) show the frequencies of populations gated in the plots with each symbol representing one mouse. Data were compiled from a single experiment. Two-tailed unpaired Student's *t*-tests were used to compare each gene-deficient group with the wild-type group; *P*-value symbols: * 0.01–0.05, *** 0.0001–0.001; **** $<0.0001$

Helios+ thymocytes that are susceptible to either fate. We show that Card11 and IL-2 signalling are essential to prevent deletion of CD4SP CCR7+ Helios+ thymocytes at distinct stages of development. Our results distinguish Card11-regulated and IL-2-regulated bottlenecks at which strongly self-reactive thymocytes undergo deletion or progress to the next stage of T-reg differentiation. The deletion prevented by IL-2 signalling is Foxp3 independent.

Although it was known that Card11 and IL-2 are necessary for thymic T-reg differentiation, the present study shows that these molecules are essential to prevent apoptotic deletion initiated by strong TCR signalling. Card11 is dispensable for a CD4SP CCR7+ thymocyte to register a strong TCR signal, but Card11 is essential to prevent apoptotic deletion in response to strong TCR signalling.[6] This interpretation is consistent with several observations in the literature. First, in strongly TCR signalled CD4SP thymocytes, T-reg differentiation occurred when the thymocytes were induced to express CD25 (a Card11-dependent event[6]), whereas deletion occurred when the thymocytes did not upregulate CD25.[23] Second, T-reg differentiation occurred when strongly TCR signalled CD4SP thymocytes could express the co-stimulatory receptor CD28, which promotes Card11-dependent CD25 upregulation in T cells,[24] whereas deletion occurred when the thymocytes

lacked CD28 expression.[25] Third, 12 h after CD4SP thymocytes were injected into a thymus expressing a deleting self-antigen, almost all of the surviving cells were CD25+.[26] We hypothesise that the outcome of the first strong TCR signalling event in a CD4SP CCR7+ thymocyte is determined by competition between a Bim-dependent pro-deletion programme and a Card11-dependent pro-survival programme. The latter programme is successful in a minority of cells, which attain a Helios+ Foxp3– phenotype. As the absence of IL-2 signalling did not decrease the CD4SP CCR7+ Helios+ Foxp3– thymocyte population, the Card11-regulated bottleneck in thymic T-reg differentiation is IL-2 independent.

The IL-2-regulated bottleneck occurs later in thymic T-reg differentiation, around the stage when CD4SP CCR7+ Helios+ Foxp3– cells upregulate Foxp3. While it was known that IL-2 is sufficient to upregulate Foxp3,[11] we show here that CD4SP CCR7+ Helios+ thymocytes that cannot respond to IL-2 are deleted, whereas wild-type cells progress to the next stage of T-reg differentiation characterised by upregulation of Foxp3 and CTLA-4. It was hypothesised that IL-2 signalling is necessary for the proliferation of cells already expressing Foxp3.[13] As most proliferating Foxp3+ cells in the thymus are mature CCR7– CD24– T-reg cells,[5] preferential loss of this mature T-reg population in the thymus may account for the decreased proliferation observed in the absence of IL-2 signalling.[13] Another hypothesis is that IL-2 signalling is required to counteract a proapoptotic protein signature induced by Foxp3.[8] This hypothesis was based, in part, on the observation that transgene-driven Foxp3 expression in total thymocytes decreases thymic cellularity, and that inhibiting apoptosis offsets this effect.[8] However, it cannot be assumed that Foxp3's proapoptotic effect in total thymocytes would also operate in cells that upregulate Foxp3 at the physiological stage of thymocyte development. The hypothesis was also based on the detection of a substantial thymic Foxp3+ population in apoptosis-defective mice that lack the common $\gamma$-chain cytokine receptor, CD132.[8] This raised the possibility that cytokine signalling may be required for thymic T-reg development only after Foxp3 is upregulated. Our findings exclude this possibility because IL-2 is still required to prevent deletion of CD4SP CCR7+ Helios+ thymocytes that are incapable of expressing Foxp3. Our data are consistent with the hypothesis that IL-2 prevents deletion initiated by strong TCR signalling.[15]

It is accepted that thymic T-reg development can pass through either a Foxp3– CD25+ or a Foxp3+ CD25– stage. The standardisation of T-reg precursor analysis based on a Foxp3– CD25+ CD4SP phenotype advanced the field considerably.[11] The identification of a thymic Foxp3+ CD25– T-reg precursor was also an advance,[27] particularly when it was shown that apoptosis defects increase preferentially the Foxp3+ CD25– population over the Foxp3+ CD25+ population.[7,8] Because CD25 upregulation was thought to mark strongly TCR signalled CD4SP thymocytes,[28] the detection of Foxp3+ CD25– thymocytes may lead to the interpretation that Foxp3 upregulation does not necessarily require a strong TCR signal. In this regard, it is notable that nascent *Bcl2l11*$^{-/-}$ Foxp3+ thymocytes are essentially all Helios+ (Figure 6). This suggests that Foxp3 upregulation in thymocytes does require TCR signalling above a certain threshold, including in the context of defective apoptosis, and that high Helios expression marks CD4SP CCR7+ thymocytes that have experienced TCR signalling above this threshold.

The alternative pathway for thymic T-reg development also attracted attention when it was shown that IL-15 was required for normal formation of Foxp3+ CD25– T-reg precursors, whereas this population remained intact when IL-2 was neutralised using anti-IL-2 antibodies.[29] This conclusion was based on experiments that used Zap70-transgenic mice, which have a sevenfold lower frequency of Foxp3+ CD25+ cells than wild-type mice.[29] Although not all reports have found an effect of IL-2 deficiency on thymic Foxp3+ cell frequency and number, a unifying finding is a marked decrease in Foxp3+ CD25+ cells.[13,14,30–32] When all thymocytes are Zap70-transgenic, the amount of IL-2 in the thymus might be lower than in wild-type mice. This would explain the greater dependence of T-reg development on IL-15[20] and the marked decrease in Foxp3+ CD25+ cells observed in the Zap70-transgenic model.[29] We were unable to detect any role for IL-15 in T-reg development when IL-2 expression was intact. Our approach should be sensitive to effects on both pathways of thymic T-reg development. In recent studies using EdU pulse labelling to identify nascent thymocytes, ~50% of nascent T-reg cells were shown to be Foxp3+ CD25– cells.[5] Therefore, the observed 80% decrease in nascent T-reg cells in thymocytes lacking CD122 (Figures 4 and 6) must involve decreases in both pathways of T-reg development. Our approach is informative in delineating steps required for CD4SP CCR7+ thymocytes to become T-reg cells. The Helios+ Foxp3– subset is susceptible to apoptotic deletion due to receipt of an above-threshold TCR signal, while Foxp3 upregulation marks the onset of IL-2 dependence in developing T-reg cells.

By distinguishing two bottlenecks regulated by Card11 and IL-2, our study draws attention to how the CD4SP thymocyte response to strong TCR signalling might mature over time. Imaging experiments have revealed that the first strong TCR signalling event is characterised by migratory arrest, but those cells that survive subsequently begin to migrate actively[33] and interact repetitively with dendritic cells (DC) within ~30 $\mu$m of their original position.[34] It was shown recently that thymic DC are an important source of IL-2,[35] which has a focal distribution in the thymic medulla.[36] Via repetitive interactions, T-reg precursors might induce local DC to produce the IL-2 that the T-reg precursors require to progress to the next stage of T-reg differentiation. If IL-2 synthesis increases with the avidity of T-reg precursor/DC interactions, it would explain why TCRs with higher avidity for self-antigen facilitate the development of larger thymic Foxp3+ T-reg cell populations.[37] We speculate that apoptotic deletion at the CD4SP CCR7+ stage of thymocyte development impinges on T-reg differentiation not by eliminating cells with too high avidity for self-antigen, but instead by eliminating cells with too low avidity for self-antigen. This hypothesis is consistent with the extremely high avidity for self-antigen observed in T-reg TCRs,[38] well above the threshold required for apoptotic deletion.[39]

## Materials and Methods

**Mice.** *Foxp3*$^{GFP}$ (*Foxp3*$^{tm1.1Ayr}$), *Foxp3*$^{null}$ (*Foxp3*$^{tm1Ayr}$), *Il2*$^{-/-}$ (*Il2tm1*$^{Hor}$), *Il15*$^{-/-}$ (*Il15*$^{tm1Imx}$), *Rag1*$^{-/-}$ (*Rag1*$^{tm1Mom}$), *Il2rb*$^{-/-}$ (*Il2rb*$^{tm1Mak}$), *Bcl2l11*$^{-/-}$ (*Bcl2l11*$^{tm1Ast}$)

and *Card11*$^{unm}$ mice have been described previously and were bred and housed at the Australian Phenomics Facility, Canberra or at Monash University, Melbourne. *Foxp3*$^{null/null}$*Rag1*$^{-/-}$ females were bred with B6 males to produce *Foxp3*$^{+/null}$ and *Foxp3*$^{null/y}$ mice. All other experimental mice were the PCR-genotyped progeny of mice heterozygous for the variant allele(s). BM from *Il2ra*$^{-/-}$ (*Il2ra*$^{tm1Dw}$) and *Il2rb*$^{-/-}$ mice was kindly provided by Dr. Jaeho Cho and Prof. Jon Sprent, Garvan Institute, Sydney. To make chimeras, recipient mice were irradiated with X-rays (two doses of 4.5 Gy given 4 h apart for *Rag1*$^{+/+}$ or one dose of 5 Gy for *Rag1*$^{-/-}$ recipients) and then injected intravenously with at least $2 \times 10^6$ bone marrow cells. The Animal Experimentation Ethics Committees of the Australian National University or Monash University approved all procedures.

**Flow cytometry.** For CCR7 staining, single-cell thymocyte suspensions were incubated for 60 min at 37 °C in pre-warmed FACS buffer (PBS containing 2% v/v heat-inactivated bovine serum and 0.01% m/v sodium azide) containing fluorochrome- or biotin-conjugated anti-CCR7 (BioLegend, San Diego, CA, USA; Cat #120104 or 120105). Cells were then pelleted by centrifugation and incubated for 30 min in FACS buffer at 4 °C containing assortments of fluorochrome-conjugated monoclonal antibodies against CD4 (BioLegend; Cat #100430), CD8$\alpha$ (BioLegend; Cat #100765), CD45.1 (BioLegend; Cat #110739), CD45.2 (BioLegend; Cat #109828). After washing in FACS buffer, cells were fixed and permeabilised using the Foxp3/Transcription Factor Staining Buffer Set (Affymetrix eBioscience, Santa Clara, CA, USA), and then incubated with antibodies specific for Helios (BioLegend; Cat #137220), CTLA-4 (eBioscience; Cat #11-5773-82) and Foxp3 (eBioscience; Cat # 11-5773-80). After washing in FACS buffer, data were acquired with LSR II flow cytometers (Becton Dickinson, Franklin Lakes, NJ, USA) and analysed using FlowJo software (FlowJo LLC, Ashland, OR, USA).

**EdU labelling.** 0.25 mg EdU was injected i.p. (0.1 ml of a 2.5 mg/ml m/v in DMSO or PBS) per mouse. After intracellular staining as described above, cells were processed following the manufacturer's instructions (Click-iT EdU Flow Cytometry Assay Kit; Thermo Fisher Scientific, Waltham, MA, USA) except that Click-iT EdU buffer additive (Component G) was used at one-fifth of the concentration recommended. Samples were then washed in FACS buffer and incubated with streptavidin-PE (BioLegend; Cat# 405204) to detect biotin-conjugated anti-CCR7.

**Cell culture.** Thymocytes from *Foxp3*$^{GFP}$ mice were stained with antibodies specific for cell surface antigens as above with the addition of anti-CD24 (BioLegend; Cat # 101822) in the 4 °C incubation. GFP– CD4SP thymocyte subsets, sorted using a FACS Aria II instrument (Becton Dickinson), were cultured in complete medium for 20 h at 37 °C in the presence or absence of graded concentrations of recombinant human IL-2 (R&D Systems, Minneapolis, MN, USA) before intracellular staining for Helios, Foxp3 and CTLA-4 as described above.

**Statistical analysis.** GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA) was used to make summary graphs and perform statistical tests as described in figure legends.

## Conflict of Interest

The authors declare no conflict of interest.

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