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Multiple molecules have been described to be important for Treg formation, maintenance and function. Indeed, one of the first to be implicated in Treg control of autoimmunity was the receptor CD28, as CD28-deficient non-obese diabetic mice have exacerbated autoimmune diabetes, which can be prevented by the transfer of Treg.[6] In 15

set out to generate mice where the loss of CD28 is restricted to the Treg lineage (*Foxp3*^{cre} *Cd28*$^{fl/fl}$). Surprisingly, this strain of mice revealed that CD28 was lost on conventional CD4 cells, in addition to Foxp3^{+} Treg, demonstrating the promiscuous expression of *Foxp3*^{cre}. Despite the loss of CD28 on a proportion of conventional CD4^{+} T cells we observed a lymphoproliferative disorder in *Foxp3*^{cre} *Cd28*$^{fl/fl}$ mice. CD28-deficient Treg were at a competitive disadvantage compared with control cells. This is due to reduced proliferation in the absence of CD28, as neither Treg lineage stability nor survival was affected by the loss of CD28 on Treg cells. Taken together, this demonstrates that CD28 expression on Tregs is important for Treg proliferation and the suppression of aberrant lymphocyte expansion.

RESULTS

FoxP3^{Cre}Cd28$^{fl/fl}$ i i 3 i

In order to determine the post-selection functions for CD28 in Foxp3^{+} Treg, we crossed the previously described Foxp3-CreYFP knockin mice[16] (thereafter referred to as *Foxp3^{Cre}*) to mice carrying a conditional CD28 allele (*Cd28$^{fl/fl}$*) derived from EUCOMM embryonic stem cells.[17] CD28 excision was monitored by surface staining for CD28, which validated Cre activity within Treg, with efficient deletion of CD28 within YFP^{+} Treg (Figures 1a and b). Unexpectedly, and in contrast to previous studies published using the *Foxp3^{Cre}* allele,[15,16,18] we also observed the deletion of CD28 from conventional CD4^{+} T cells (Figures 1a and b). Deletion was observed in both naïve and, to a lesser extent, antigen-experienced conventional T cells, with considerable variation in the level of deletion (Figures 1b and c), indicative of low-level stochastic Cre activation in non-Tregs or a lineage precursor. Notably, the level of CD28 excision is higher in naïve T cells than in antigen-experienced CD62L^{low} T cells (Figure 1b), consistent with the function of CD28 in allowing transition between these activation states. Furthermore, we observed an indirect correlation between the level of non-specific CD28 excision and the frequency of CD4^{+}CD25^{+} T cells (Figure 1c). As CD28-deficient T cells are poor IL-2 producers,[11,19] this reduction in Treg numbers in mice with high levels of non-specific CD28 excision is likely due to impaired IL-2-mediated Treg homeostasis.[14]

Despite the promiscuous activity of Cre, which should be considered when using *Foxp3^{Cre}* mice, we assessed the function for CD28 in Treg after selection. Male *Foxp3^{Cre}Cd28$^{fl/fl}$* mice developed a lymphoproliferative disorder, including lymphadenopathy, mild splenomegaly, T-cell activation and lymphocytic organ infiltrations (Figures 2a and b and data not shown). In rare cases, mice presented with disease progression similar to scurfy mice (with fatal disease at 4 weeks of age). However, disease onset and severity were highly variable (Figures 2c and d), with other mice showing a milder phenotypic appearance, similar to the observations of Turka and colleagues.[15] Correlation between the degree of T-cell activation and promiscuous *Cd28* excision (Figure 2e) indicates that lineage-specific Treg excision resulted in severe disease, whereas promiscuous *Cd28* excision resulted in the milder form of disease, probably due to the intrinsic defect in T-cell activation caused by the loss of CD28 (akin to the *Cd28* knockout mouse).[19]

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Despite the variability in the disease caused by non-specific Cre activity in *Foxp3^{Cre} Cd28$^{fl/fl}$* mice, we were able to assess the impact of *Cd28* excision in Treg by limiting analysis to female *Foxp3$^{Cre/wt}$Cd28$^{fl/fl}$* mice, where X chromosome inactivation restricts Cre activity to 50% of the hematopoietic compartment. In these mice, wild-type and *Cd28*-deleted Treg are generated in equal proportions and survive within the same microenvironment. In *Foxp3$^{Cre/wt}$Cd28$^{fl/fl}$* mice, CD28-deficient Treg registered a profound competitive disadvantage, with a relative decrease in number of CD28-deficient Treg cells from the default 50 to ~20% of the total Treg (Figure 3). These results demonstrate that post-Treg selection CD28 expression is critical for homeostasis, indicating a function either in lineage stability, proliferation or apoptosis.

As a serendipitous observation, we found that CD28-deficient Treg in female *Foxp3$^{Cre/wt}$Cd28$^{fl/fl}$* mice had reduced Foxp3 protein expression when compared with wild-type Treg in the same mice (Figure 4a). However, a similar reduction was observed also in *Foxp3^{Cre}Cd28$^{+/+}$* Treg when co-stained with congenically marked wild-type Treg (Figure 4b). Therefore, decreased Foxp3 expression was not caused by the loss of CD28 signalling and rather represents a mild hypomorphic feature of the *Foxp3^{Cre}* allele. Despite the false lead of depressed Foxp3 expression, the *Foxp3* locus contains a CD28 response element in the conserved non-coding DNA sequence 3,[20] which still allowed for the possibility that CD28 directly modulates the stability of Foxp3 expression, and hence the stability of the Treg lineage. We therefore investigated whether the loss of CD28 signalling would result in reduced Treg lineage stability and whether ex-Foxp3^{+} cells, such as described by Miyao *et al.*,[21] contributed to the above-described population of CD28-deficient non-Treg. To this end, Treg

F . 1 Foxp3^{Cre} activity is not restricted to Foxp3^{+} Treg. (, .) Lymph node CD4^{+} lymphocytes were analysed for the presence of CD28cells among YFP^{+} Treg and YFP^{-} non-Treg in Foxp3^{Cre}Cd28$^{fl/fl}$ males. ( ) representative flow cytometric analysis for CD28^{-} CD4 T cells is shown. (.) The plot summarises three independent experiments with a total of n = 9 (Treg) and n = 6 (CD62L^{+} and CD62L^{-} non-Treg); mean ± s.e.m. ( ) The graph depicts the inverse relationship between the presence of CD28non-Treg and CD25^{-} Treg in Foxp3$^{Cre/wt}$Cd28$^{fl/fl}$ females. Graph represents six independent experiments with a total of n =

from *Foxp3*^{Cre}*CD28*$^{fl/fl}$ and *Foxp3*^{Cre}*Cd28*$^{+/+}$ mice were purified and the methylation status of the Treg-specific demethylated region (TSDR) was analysed (Figure 4c). The data showed complete demethylation of the Treg-specific demethylated region in the presence and absence of CD28 indicative of stable Foxp3 expression.

CD28-sufficient Treg (Figures 5a and b). To exclude the possible influence of the hypomorphic *Foxp3*^{Cre} allele on Treg homeostasis, we analysed the proliferation status of *Foxp3*^{Cre}*Cd28*$^{+/+}$ Treg and found

CD28. Here we addressed the peripheral requirement of CD28

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