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c-Rel employs multiple mechanisms to promote the thymic development and peripheral function of regulatory T cells in mice

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## **Keywords**

c-Rel, Regulatory T cells, Thymic development, Cell cycle progression

## **Abbreviations used**

Treg – regulatory T cell

Pre-Treg – regulatory T cell precursors

tTreg – thymically derived regulatory T cell

pTreg – peripherally derived regulatory T cell

iTreg – *in vitro* induced regulatory T cell

cTreg – central or naïve regulatory T cell

eTreg – effector or activated regulatory T cell

Tcon – conventional T cell

TCR – T cell receptor

pLN – peripheral lymph nodes; pooled subcutaneous lymph nodes comprising inguinal, axial, brachial and superficial cervical

Ab – Antibody

## ABSTRACT

The NF- $\kappa$ B transcription factor c-Rel is a critical regulator of regulatory T cell (Treg) ontogeny, controlling multiple points of the stepwise developmental pathway. Here, we found that the thymic Treg development defect in c-Rel-deficient (*cRel*<sup>-/-</sup>) mice is quantitative not qualitative based on analyses of T cell receptor (TCR) repertoire and TCR signalling strength. However, these parameters were altered in the thymic Treg-precursor population, which is also markedly diminished in *cRel*<sup>-/-</sup> mice. Moreover, c-Rel governs the transcriptional programme of both thymic and peripheral Tregs, controlling a core of genes involved with immune signalling, and separately in the periphery, cell cycle progression. Lastly, the immune suppressive function of peripheral *cRel*<sup>-/-</sup> tTregs is diminished in a lymphopenic model of T cell proliferation and is associated with decreased stability of Foxp3 expression. Collectively, we show that c-Rel is a transcriptional regulator that controls multiple aspects of Treg development, differentiation and function via distinct mechanisms.

## INTRODUCTION

Regulatory CD4<sup>+</sup> T cells (Tregs) expressing the transcription factor Forkhead box P3 (Foxp3) are critical for maintaining immune homeostasis by restraining abnormal immune responses and suppressing auto-reactive T cells [1-3]. The majority of Tregs in lymphoid organs develop in the thymus (tTregs) from precursors with high affinity for self-antigens [4-6]. However, Foxp3 expression may also be induced in conventional CD4<sup>+</sup> T cell (Tcon) that have left the thymus, producing peripheral Tregs (pTregs), or in culture following T cell receptor (TCR) and TGF- $\beta$  engagement to produce induced Tregs (iTregs). Each Treg subset has immune suppressive properties and complementary functions [7, 8]. Similar to CD4<sup>+</sup> Tcon, circulating Tregs cells also comprise many sub-populations with distinct functions and properties [9-11]. Most Tregs in peripheral lymphoid organs have a naïve, or central (cTreg), phenotype typically defined by high levels of L-selectin (CD62L) and lower expression of T cell activation markers such as CD44, ICOS and GITR [11-13]. Conversely, a smaller proportion of Tregs in lymphoid tissues, and most Tregs in non-lymphoid organs, have an activated or effector-like phenotype (eTregs) and are thought to have enhanced suppressive function [11-13].

The diverse properties of different sub-populations reflect the various transcriptional programmes instructed in Treg cells. While Foxp3 is essential for the immune suppressive properties of Tregs, alone it is unable to maintain these functions [14-16]. Instead, Foxp3 functions by stabilizing transcriptional programmes created through the combined activity of other transcription factors during Treg development [17-19]. The development of *de novo* thymic Tregs occurs as a step-wise process that has been proposed to first involve pre-Treg-precursors [20], CD4<sup>+</sup>CD122<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>-</sup> thymocytes, that then become CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>hi</sup>GITR<sup>hi</sup>Foxp3<sup>-</sup> Treg precursors (pre-Treg) following T cell receptor (TCR) and co-

stimulatory signals, after which Foxp3 is stably induced in response to IL-2 and/or IL-15 [21, 22] locking-in the pre-existing transcriptional landscape [18, 23]. Following thymic egress, the basic transcriptional blueprint that defines a naïve Treg is further refined during eTreg differentiation by transcription factors such as T-bet, Irf4, Gata3, Ror $\gamma$ t and Bcl6, which are essential for the differentiation of Treg populations that can control specific conventional CD4<sup>+</sup> T helper (Th) subsets [24-29].

Among the various transcriptional regulators controlling Treg development and differentiation are the canonical NF- $\kappa$ B factors, proteins comprising homodimers or heterodimers of RelA, c-Rel and NF- $\kappa$ B1 [30-32]. c-Rel is the most important NF- $\kappa$ B protein during thymic Treg development, with its absence resulting in a loss of ~85% of the Treg precursors (pre-Tregs) and tTregs [33-38]. RelA serves a minor role in tTreg development, while NF- $\kappa$ B1 appears to be dispensable [33, 39]. Notwithstanding its major role in thymic Treg development, the impact c-Rel has on peripheral function and homeostasis are poorly understood. Conditional targeting of upstream regulators of the canonical NF- $\kappa$ B pathway in T cells confirms that Tregs require NF- $\kappa$ B activity to maintain peripheral tolerance [40-44]. This peripheral requirement for NF- $\kappa$ B in part depends on RelA [39, 45], although the heightened severity of the IKK $\beta$  knockout in particular compared to that of RelA-deficient Tregs, points to other canonical NF- $\kappa$ B proteins such as c-Rel contributing to peripheral Treg function.

Here we undertake a comprehensive survey of how c-Rel controls tTreg development and its subsequent impact on peripheral Treg biology. c-Rel is important for controlling the size of the thymic pre-Treg population by providing survival signals during clonal deletion but not selection of high affinity TCRs. We also provide evidence that the activation of c-Rel during the pre-Treg to Treg transition, and its putative role in controlling Foxp3 induction, is linked

to GITR-augmented IL-2 signalling. Importantly, a shared core of genes is controlled by c-Rel in both thymic and peripheral tTregs, confirming that c-Rel is responsible for establishing a subset of the Treg transcriptional landscape. Further analysis of peripheral Tregs found that eTregs were under-represented in *cRel*<sup>-/-</sup> mice, consistent with the preferential activation of c-Rel in this Treg subset. While c-Rel was found to be dispensable for eTreg differentiation *per se*, including expression of the effector Treg master regulator Blimp, the homeostatic proliferation of *cRel*<sup>-/-</sup> Tregs was impaired. Finally, an examination of the immune suppressive capacity of peripheral *cRel*<sup>-/-</sup> tTregs revealed that these cells are less effective at restraining conventional T cell proliferation and exhibit increased instability associated with a loss of Foxp3 in a lymphopenic environment. Collectively this study firmly establishes c-Rel as a transcriptional regulator that controls multiple aspects of Treg development, differentiation and function via distinct mechanisms.

## RESULTS

### **c-Rel is present in the nucleus of pre-Tregs and newly developed thymic Tregs**

We have shown previously that c-Rel is critical for thymic Treg development (Fig. 1A) [33]. To better understand the relationship between c-Rel activity and tTreg development, electrophoretic mobility gel shift assays were conducted on nuclear extracts prepared from *ex vivo* purified pre-Tregs [CD4<sup>+</sup>CD25<sup>hi</sup>GITR<sup>hi</sup>Foxp3(RFP)<sup>-</sup>] and newly generated CCR7<sup>+</sup> thymic Tregs [CD4<sup>+</sup>CD25<sup>hi</sup>GITR<sup>+</sup>Foxp3(RFP)<sup>+</sup>] (Figs. 1B-D and Sup. 2). Pre-Tregs exhibited nuclear NF-κB activity (Fig. 1B, first lane) that included c-Rel (Fig. 1C). A similar pattern of nuclear NF-κB, including c-Rel expression was observed in thymic CCR7<sup>+</sup> Tregs (Fig. 1B, second lane; 1D). These findings indicate that c-Rel is in an active state prior to and following the induction of Foxp3 in thymocytes disposed to becoming Tregs.

### **c-Rel is not required for thymocytes to register a strong TCR signal**

To understand why the pre-Treg population size is reduced in *cRel*<sup>-/-</sup> mice, we assessed whether c-Rel affects the capacity of CD4<sup>+</sup>CD8<sup>-</sup> single-positive (CD4SP) thymocytes to register a strong TCR signal. In the 3A9 x insHEL double-transgenic model, insHEL recognition induces strong TCR signalling in CD4SP thymocytes which then undergo clonal deletion or Treg differentiation [46]. While the absence of c-Rel markedly reduced the frequency of insHEL-specific (TCR<sup>3A9+</sup>) Foxp3<sup>+</sup> Tregs, as expected, it did not impair the insHEL-mediated reduction of TCR<sup>3A9+</sup>CD4<sup>+</sup> Tcon in the thymus or spleen (Fig. Sup. 3A-E). CD25<sup>+</sup>Foxp3<sup>-</sup> thymic pre-Treg tended to be reduced in frequency in *cRel*<sup>-/-</sup> 3A9 x insHEL mice, although this was not statistically significant (Fig. Sup. 3C). These findings suggest self-reactive CD4<sup>+</sup> T cells can receive a strong TCR signal and adopt a pre-Treg phenotype in *cRel*<sup>-/-</sup>



<sup>-/-</sup> mice, but most such cells are physically eliminated before reaching the Foxp3 upregulation step in thymic Treg development.

### **Certain abnormalities are detectable within *cRel*<sup>-/-</sup> pre-Tregs, but not *cRel*<sup>-/-</sup> thymic Tregs**

Thymic Tregs typically express TCRs with a higher affinity for self-antigens than conventional CD4<sup>+</sup> T cells [4-6, 47, 48], with pre-Treg development intimately associated with the receipt of stronger TCR signals [20, 21, 37]. To test whether the absence of c-Rel affects TCR signalling in developing Treg cells, we used a *Nur77*<sup>gfp</sup> transgenic reporter mouse [48] in which GFP expression correlates with TCR signal strength (*cRel*<sup>-/-</sup>*Foxp3*<sup>gfp</sup>*Nur77*<sup>gfp</sup>). While the MFI of Nur77(GFP) was normal in Tcon and thymic Tregs from *cRel*<sup>-/-</sup> mice, this parameter was subtly but significantly reduced in *cRel*<sup>-/-</sup> pre-Tregs, compared to *cRel*<sup>+/+</sup> counterparts (Fig. 2A). We excluded the possibility that c-Rel is itself required for the induction of Nur77, as the absence of c-Rel did not affect Nur77(GFP) induction in CD4<sup>+</sup>Foxp3<sup>-</sup> Tcon stimulated with various concentrations of anti-CD3 and a fixed concentration of anti-CD28 (Fig. Sup. 3F).

To assess whether the absence of c-Rel affects the Treg TCR repertoire, we sequenced TCRα chains expressed by thymic pre-Treg and Treg populations purified from mice expressing the Yae62 TCRβ transgene (Yae62β-tg) [49]. The presence of certain, predominantly hydrophobic, amino acids at positions 6 and 7 of the complementarity-determining region 3 (CDR3) promotes T cell self-reactivity [50]. Compared to *cRel*<sup>+/+</sup> counterparts, the frequency of unique sequences with hydrophobic doublets (the hydrophobic index) was normal in *cRel*<sup>-/-</sup> thymic Tregs, but subtly decreased in *cRel*<sup>-/-</sup> pre-Tregs (Fig. 2B). We also assessed the extent of TCR sharing between our dataset and a published dataset from wild-type Yae62β-tg mice [51]. In thymic Tregs, the extent of overlap with the published Treg TCR catalogue was similar

for the *cRel*<sup>+/+</sup>, *cRel*<sup>+/-</sup> and *cRel*<sup>-/-</sup> groups, indicating that c-Rel deficiency does not detectably change the thymic Treg TCR repertoire. However, compared to *cRel*<sup>+/+</sup> controls, the *cRel*<sup>+/-</sup> and *cRel*<sup>-/-</sup> pre-Treg TCR repertoires showed greater overlap with the published CD4<sup>+</sup> Tcon TCR dataset (Fig. 2C). Thus, the changes in Nur77(GFP), the hydrophobic index and TCR sharing converge on the pre-Treg stage as being altered by c-Rel deficiency, whereas these abnormalities were not detected in the thymic Treg population. Rarefaction and extrapolation curves based on the observed number of TCR $\alpha$  clones (Fig. 2D, left) were converted to curves based on sample coverage (Fig. 2D, middle) to enable the comparison of *Trav*–*Traj* diversity at an equal level of completeness [52, 53]. The diversity of *Trav*–*Traj* combinations in thymic pre-Treg and Treg TCR $\alpha$  repertoires were normal in *cRel*<sup>-/-</sup> mice (Fig. 2D, right and data not shown).

We also surveyed the expression of cell surface markers normally upregulated during pre-Treg development [54]. Notably, expression of the TNF receptor super family (TNFRSF) members GITR, OX40 and TNFR2 were all significantly lower on *cRel*<sup>-/-</sup> pre-Tregs (Fig. 3A). To determine whether additional changes in pre-Tregs coincide with a lack of c-Rel, RNA-seq transcriptome analysis was conducted on *cRel*<sup>+/+</sup> and *cRel*<sup>-/-</sup> CD4<sup>+</sup>CD25<sup>hi</sup>GITR<sup>+</sup>Foxp3<sup>-</sup> pre-Tregs. Surprisingly, the gene expression patterns in *cRel*<sup>+/+</sup> and *cRel*<sup>-/-</sup> pre-Tregs were highly similar, with only nine genes exhibiting a significantly altered pattern of expression (Fig. 2E). *Tnfrsf18* (*Gitr*), *Tnfrsf1b* (*Tnfr2*) and *Tnfrsf4* (*Ox40*) mRNA levels were reduced in *cRel*<sup>-/-</sup> pre-Tregs, as expected based on protein expression, however the differences when compared to *cRel*<sup>+/+</sup> pre-Tregs were not significant. Collectively, these results demonstrate that despite a marked reduction in *cRel*<sup>-/-</sup> pre-Treg numbers, nuclear c-Rel activity does not exert a major influence on the transcriptional programme of pre-Treg cells.

### **GITR co-stimulation fails to augment the induction of Foxp3 in pre-Tregs lacking c-Rel**

Compared to wild-type controls, a reduced percentage of c-Rel-deficient CD4SP Foxp3<sup>-</sup> thymocytes or pre-Tregs, up-regulate Foxp3 when cultured with IL-2 or IL-15 [37, 38]. Recently, it was shown that engagement of the TNF receptor superfamily (TNFRSF) members, GITR, OX40 and TNFR2 augments the IL-2-mediated induction of Foxp3 in pre-Tregs [54]. Given these TNFRSF members are reduced on *cRel*<sup>-/-</sup> pre-Tregs, we assessed the TNFRSF-mediated augmentation of IL-2 induced Foxp3 expression in cultured pre-Tregs. Foxp3 induction was compared in *cRel*<sup>+/+</sup> and *cRel*<sup>-/-</sup> CD4<sup>+</sup>CD25<sup>hi</sup>Foxp3(RFP)<sup>-</sup> pre-Tregs incubated for 24 h with a 100-fold range of IL-2 concentrations in the presence of soluble recombinant GITRL. GITRL enhanced the ability of IL-2 to upregulate Foxp3 in *cRel*<sup>+/+</sup> pre-Tregs, but not in *cRel*<sup>-/-</sup> pre-Tregs (Fig. 3B), even when using higher concentrations of GITRL (Fig. Sup. 4A). In contrast to the findings of Mahmud *et al.* (2014), soluble recombinant OX40L was unable to augment the IL-2 induction of Foxp3 in either cultured *cRel*<sup>+/+</sup> or *cRel*<sup>-/-</sup> pre-Tregs (Fig. Sup. 4B).

The involvement of c-Rel in the GITRL/GITR-dependent induction of Foxp3 in pre-Tregs raised the possibility that in addition to a pre-Treg intrinsic role, c-Rel-dependent defects in APCs may also contribute to the impaired generation of Tregs in *cRel*<sup>-/-</sup> mice. To assess this possibility, thymic Treg development was examined in mice that lack c-Rel only in T lineage cells. Floxed c-Rel mice [55] expressing a *Lck*<sup>cre</sup> transgene (*Lck*<sup>cre</sup>*cRel*<sup>fl/fl</sup>) revealed that a T cell restricted absence of c-Rel generated a pre-Treg and Treg profile akin to that observed in *cRel*<sup>-/-</sup> mice (Fig. 3C), and additionally display a similar decrease in GITR expression on developing Tregs (Fig. Sup. 4C). This finding is also in agreement with our previous work where mixed bone marrow chimeras revealed a defect in thymic Treg numbers in the *cRel*<sup>-/-</sup> compartment [33], suggesting that the provision of IL-2 by wild-type thymic APCs or by-stander T cells is

not sufficient to overcome the block in thymic Treg development resulting from a lack of c-Rel. Collectively these *in vitro* and *in vivo* findings confirm a critical T cell intrinsic requirement for c-Rel in the induction of Foxp3, and that one mechanism by which c-Rel contributes to IL-2-dependent induction of Foxp3 appears to be by modulating GITR expression and function.

### ***cRel*<sup>-/-</sup> mice display reduced thymic populations of effector-like Tregs**

In adult mice, the thymic Treg population comprises a heterogeneous mix of sub-populations. This includes newly generated (*de novo*) CCR7<sup>+</sup>CCR6<sup>-</sup> Tregs, and a mature CCR7<sup>-</sup>CCR6<sup>+</sup> Treg population that appears to result from recirculation back, and/or retention of, Tregs in the thymus [56, 57]. The proportion of mature Tregs in the thymus increases with age and reaches 50%-80% of the Treg compartment in an adult mouse thymus [58]. An examination of *cRel*<sup>+/+</sup> and *cRel*<sup>-/-</sup> mice revealed that irrespective of age, the frequency of mature CCR6<sup>+</sup> Tregs was significantly lower in *cRel*<sup>-/-</sup> thymi (Fig. 4A). Tregs re-circulating through the thymus generally display an activated or effector Treg (eTreg) phenotype, as indicated by the high levels of CCR6 expression [58]. Cells expressing phenotypic markers of eTregs, which include low levels of CD62L plus high levels of CD44 (CD44<sup>hi</sup>CD62L<sup>lo</sup>), as well as ICOS, TIGIT and CD103 expression, were significantly under-represented in *cRel*<sup>-/-</sup> mice (Figs. 4B and Sup. 5). To ascertain if this change is due to T cell intrinsic or extrinsic factors, the CCR7<sup>+</sup> to CCR6<sup>+</sup> Treg ratio was also compared in 6 wk old *Lck<sup>cre</sup>Rel<sup>wt/wt</sup>* and *Lck<sup>cre</sup>Rel<sup>fl/fl</sup>* mice (Fig. 4C). Like *cRel*<sup>-/-</sup> mice, the frequency of CCR6<sup>+</sup> thymic Tregs was still markedly lower in *Lck<sup>cre</sup>Rel<sup>fl/fl</sup>* mice, indicating that the reduced thymic CCR6<sup>+</sup> Treg population is likely a T cell-intrinsic defect.

### **The absence of c-Rel alters gene expression in Foxp3<sup>+</sup>CCR7<sup>+</sup> thymic Tregs**

c-Rel involvement in GITR-augmented Foxp3 induction raised the possibility that c-Rel might serve a broader role regulating gene expression during the pre-Treg to Treg transition. This was examined using RNA-seq to compare the transcriptome of newly generated CD4<sup>+</sup>CCR7<sup>+</sup>CD25<sup>hi</sup>GITR<sup>+</sup>Foxp3<sup>+</sup> thymic Tregs from 6 wks old *cRel*<sup>+/+</sup>*Foxp3*<sup>flp</sup> and *cRel*<sup>-/-</sup>*Foxp3*<sup>flp</sup> mice. Unlike the minimal changes in gene expression seen in *cRel*<sup>-/-</sup> pre-Tregs, we observed a marked disruption in the transcriptome of thymic Tregs in c-Rel-deficient mice. 735 significant differences in gene expression were seen in *cRel*<sup>-/-</sup> CCR7<sup>+</sup> Tregs, of which 343 genes were upregulated and 392 down regulated when compared to *cRel*<sup>+/+</sup> cells (Fig. 5A). Of the nine genes altered in *cRel*<sup>-/-</sup> pre-Tregs, eight were also altered in *cRel*<sup>-/-</sup> thymic Tregs (Fig. 5B). Pathway analysis using the Ingenuity program revealed that the main functions impacted by the gene expression changes seen in CCR7<sup>+</sup> *cRel*<sup>-/-</sup> thymic Tregs impinge upon T helper cell activation and differentiation, with protein synthesis and translation, apoptotic signalling and leukocyte extravasation also significantly changed by the loss of c-Rel (Fig. 5C). Amongst the altered genes were 33 known transcription factors. Notable amongst these were 8 transcriptional co-factors (Fig. 5D) previously shown to interact with Foxp3 to establish the Treg gene signature [12, 23, 59]. Of these, *Ahr* [60], *Irf4* [61], *Stat5a* [62] and *Ikzf4* [38], are known or putative NF-κB target genes. These findings suggest that c-Rel controls thymic Treg development in part by regulating the expression of transcription factors that co-ordinate Foxp3-dependent transcription.

### **The influence of c-Rel on peripherally derived Tregs depends on anatomical location**

The importance of c-Rel in tTreg development is well established, however, its role in pTreg generation and function is largely unknown. The sub-division of splenic and pooled subcutaneous lymph node (pLN; comprising inguinal, axillary, brachial and superficial cervical lymph nodes) Tregs into Nrpl<sup>+</sup> tTreg and Nrpl<sup>-</sup> pTreg cells [63, 64] revealed that the frequency

of Nrp1<sup>+</sup> pTregs is significantly higher in *cRel*<sup>-/-</sup> mice. However, due to the difference in overall Treg population size, similar numbers of Nrp1<sup>+</sup> pTregs were detected in the spleen and pLN of *cRel*<sup>+/+</sup> and *cRel*<sup>-/-</sup> mice (Figs. 6A and Sup. 6A). By contrast, Nrp1<sup>+</sup> pTreg numbers in the liver were significantly lower in mice lacking c-Rel, indicating that the impact of c-Rel on the pTreg population is greater at anatomical sites enriched for pTregs [65].

To understand how c-Rel activity might impact pTregs, iTreg conversion assays were performed using naïve [CD44<sup>lo</sup>CD25<sup>lo</sup>Foxp3(RFP)-] *cRel*<sup>+/+</sup> and *cRel*<sup>-/-</sup> splenic CD4 T cells. Initially, different concentrations of anti-CD3 antibody (Ab) and a fixed concentration of anti-CD28 Ab were used in conjunction with soluble TGF-β to assess the impact of c-Rel on iTreg generation [66, 67]. Although the number of *cRel*<sup>-/-</sup> iTregs was markedly lower at all anti-CD3 Ab concentrations, the frequency of *cRel*<sup>-/-</sup> Foxp3<sup>+</sup> cells was only reduced at low (0.5 and 1 μg/mL) antibody concentrations (Fig. 6B). Moreover, the viability of *cRel*<sup>-/-</sup> Foxp3<sup>+</sup> cells was lower at 0.5 μg/mL of anti-CD3 Ab, but otherwise only slightly reduced at higher anti-CD3 Ab concentrations (Fig. 6C). Thus, the difference in *cRel*<sup>+/+</sup> and *cRel*<sup>-/-</sup> iTreg numbers was mainly due to reduced proliferation of *cRel*<sup>-/-</sup> iTregs. This was evident by the mean cellular division of *cRel*<sup>-/-</sup> T cells that induced Foxp3 as assessed by CellTrace Violet dilution (Fig. 6D). While c-Rel can be activated in conventional T cells by CD28 signals [68], increasing the anti-CD28 Ab levels did not enhance iTreg generation in *cRel*<sup>-/-</sup> cells (Fig. Sup. 6B). These findings indicate that c-Rel contributes to iTreg generation by promoting the TCR-dependent proliferation of conventional CD4 T cells prior to or following the induction of Foxp3.

We have shown previously that c-Rel-deficient T cells have a reduced capacity to proliferate in response to TCR stimulation due to a defect in IL-2 production [69]; specifically, the addition of exogenous IL-2 restores the proliferative response of *cRel*<sup>-/-</sup> T cells in response to

anti-CD3/anti-CD28 stimulation. Therefore, we examined the effect of exogenous IL-2 on the ability of *cRel*<sup>-/-</sup> naïve CD4<sup>+</sup> T cells to proliferate and up-regulate Foxp3 in response to TCR and TGF-β signals in culture. Foxp3 induction in *cRel*<sup>-/-</sup> naïve CD4<sup>+</sup> T cells was restored by the addition of 1 ng/mL IL-2 (Fig. 6E), suggesting the reduced responsiveness of *cRel*<sup>-/-</sup> T cells to lower concentrations of anti-CD3 antibody is due to a deficiency in the production of IL-2, which in turn limits proliferative responses and/or the induction of Foxp3 in T cells in culture.

### **Effector Tregs are under-represented in peripheral *cRel*<sup>-/-</sup> Treg populations**

Analysis of activated/effector Tregs in *cRel*<sup>+/+</sup> and *cRel*<sup>-/-</sup> mice based on CD44 and CD62L expression found that the frequencies of CD44<sup>hi</sup>CD62L<sup>lo</sup> eTregs cells were similar in the spleen, whereas the frequency of CD44<sup>hi</sup>CD62L<sup>lo</sup> eTregs in *cRel*<sup>-/-</sup> pLNs was elevated (Fig. 7A). By contrast, the frequency of *cRel*<sup>-/-</sup> Tregs that expressed high levels of other eTreg markers, including ICOS, CD103 and TIGIT were all significantly lower in the spleen (Figs. 7A and Sup. 7A). Given eTregs are highly enriched in non-lymphoid tissues, SI, lung, liver and visceral adipose tissue (VAT) were examined with the frequency of total Tregs at these peripheral sites also markedly reduced in *cRel*<sup>-/-</sup> mice (Fig. 7B). Collectively, these findings indicate that an absence of c-Rel leads to a reduction in the frequency of Tregs with effector function. To explore NF-κB activity in peripheral Treg sub-populations, gel shift assays were performed on nuclear extracts isolated from splenic cTregs (ICOS<sup>lo</sup>) and eTregs (ICOS<sup>hi</sup>) (Fig. 7C). Low levels of NF-κB were detected in cTregs, whereas two distinct NF-κB/DNA complexes were present in eTregs. Antibody super shifts revealed that C1 consisted of NF-κB1 homodimers, whereas C2 was a mix of NF-κB proteins of similar mobility comprising heterodimers of c-Rel or RelA and NF-κB1. This indicates that a higher level of c-Rel activity in peripheral Tregs is associated with an activated/effector phenotype.

Whether the lower number of eTregs in *cRel*<sup>-/-</sup> mice reflects a defect in eTreg differentiation, homeostasis, or both, was unclear. An examination of the capacity of *cRel*<sup>+/+</sup> and *cRel*<sup>-/-</sup> cTregs (CD44<sup>lo</sup>ICOS<sup>lo</sup>Foxp3<sup>+</sup>) to upregulate CD44 and ICOS following TCR and IL-2 activation in culture over 3 days showed that these markers were able to be induced similarly by *cRel*<sup>+/+</sup> and *cRel*<sup>-/-</sup> cTregs (Fig. Sup. 7B). Given the Blimp transcription factor is a key regulator of eTreg differentiation and function [10, 11], a Blimp(GFP) (*Prdm1*<sup>gfp</sup>) reporter strain [70] was used to independently quantify eTregs in *cRel*<sup>-/-</sup> mice. Compared to controls, the frequency of Blimp(GFP)<sup>+</sup> splenic Tregs in 12 wk old *cRel*<sup>-/-</sup>*Foxp3*<sup>flp</sup>*Prdm1*<sup>gfp</sup> mice tended to be reduced, although this trend was not statistically significant (Fig. 7D). As expected, Blimp(GFP) expression was associated with the expression of ICOS in both *cRel*<sup>+/+</sup> and *cRel*<sup>-/-</sup> splenic Tregs (Fig. Sup. 7C). Collectively, these data indicate that c-Rel is dispensable for generating an activated eTreg-like phenotype, although the frequency of eTregs is disproportionately diminished in c-Rel-deficient mice.

### **Splenic Tregs lacking c-Rel exhibit a proliferative defect**

While the frequency of splenic eTregs as defined by Blimp expression appears normal, the impact of c-Rel on the properties of peripheral Tregs including effector functions was unclear. To determine which biological pathways c-Rel controls in peripheral tTregs, the transcriptomes of splenic *cRel*<sup>+/+</sup> and *cRel*<sup>-/-</sup> CD4<sup>+</sup>Nrp1<sup>+</sup>CD25<sup>hi</sup>Foxp3<sup>+</sup> tTregs were compared using RNA-seq analysis. The expression of 246 genes differed significantly (113 and 133 down-regulated and up-regulated respectively) in *cRel*<sup>-/-</sup> Tregs (Fig. 8A). Consistent with Blimp-dependent eTreg differentiation occurring in the absence of c-Rel, a cohort of 200 Blimp-associated eTreg genes [71] was found to be largely intact (Fig. 8B). Comparing genes differentially expressed in both thymic and splenic *cRel*<sup>-/-</sup> Tregs identified a common group of 97 genes (Fig. 8C), of which 89 exhibited the same trend in altered expression (increased or decreased compared to *cRel*<sup>+/+</sup>



cells). This core of differentially expressed transcripts whose expression is altered in both thymic and splenic *cRel*<sup>-/-</sup> Tregs feature genes involved with immune cell signalling, T helper cell differentiation, antigen presentation and apoptotic signalling pathways (Fig. Sup. 8). Conversely, Ingenuity pathway analysis revealed that amongst those genes altered in *cRel*<sup>-/-</sup> splenic Tregs there was a strong enrichment of genes involved in the cell cycle and DNA damage repair (Fig. 8D). In particular, amongst the 113 genes expressed at lower levels in *cRel*<sup>-/-</sup> splenic Tregs, ~ 50% encoded cell cycle proteins, with these genes enriched for proteins involved in the regulation of S, G2 and M phases of the cell cycle. The reduced division of *cRel*<sup>-/-</sup> Nrp1<sup>+</sup> splenic tTregs was independently verified by Ki67 staining (Fig. 8E). Collectively, these findings indicate that c-Rel plays an important role controlling the proliferation of peripheral Tregs but not an intrinsic capacity of *cRel*<sup>-/-</sup> Tregs to adopt an effector phenotype.

### **TCR diversity, stability and immune suppressive function of peripheral *cRel*<sup>-/-</sup> Tregs is diminished**

The consequences of c-Rel deficiency on the peripheral Treg TCR repertoire are incompletely understood. The Nur77(GFP) MFI detected in splenic *cRel*<sup>-/-</sup> Nrp1<sup>+</sup> tTregs was reduced (Fig. 9A), suggesting that tonic TCR signalling in peripheral Tregs is decreased. TCR $\alpha$  sequencing of Nrp1<sup>+</sup> splenic Tregs in Yae62 $\beta$ -tg mice revealed no effect of c-Rel deficiency on the hydrophobic index of self-reactivity (Fig. 9B) or on the extent of overlap with a published database of Tcon TCR $\alpha$  sequences (Fig. 9C) [51]. However, the diversity of *Trav-Traj* combinations used by splenic Tregs was subtly reduced in *cRel*<sup>-/-</sup> Yae62 $\beta$ -tg mice (Fig. 9D). These findings raise a possibility whereby a normal core Treg TCR repertoire is maintained in the absence of c-Rel, yet overall TCR diversity is detectably reduced in peripheral Treg.

We previously reported that *cRel*<sup>-/-</sup> Tregs effectively suppress T cell proliferation in culture [33]. However, the use of cultured Tregs is not necessarily an accurate gauge of immune suppressive activity *in vivo* [72], so we assessed the ability of *cRel*<sup>-/-</sup> splenic Tregs to inhibit the lymphopenia-induced expansion of conventional CD4<sup>+</sup> T cells adoptively transferred into *Rag1*<sup>-/-</sup> mice, as previously described [39, 73]. Peripheral Tregs (Ly5.2<sup>+</sup>) lacking c-Rel were less effective than *cRel*<sup>+/+</sup> Tregs at limiting the expansion of conventional Ly5.1<sup>+</sup>CD4<sup>+</sup> T cells in the spleen and pLNs, but not mesenteric LNs (mLN), following co-transfer in to *Rag1*<sup>-/-</sup> mice (Fig. 10A). Analysis of the Treg population in these *Rag1*<sup>-/-</sup> recipients after 4 weeks revealed that the total number of splenic Tregs [CD4<sup>+</sup>CD25<sup>hi</sup>Foxp3(RFP)<sup>+</sup>] was significantly lower in *Rag1*<sup>-/-</sup> mice receiving *cRel*<sup>-/-</sup> Tregs (Fig. Sup. 9). Furthermore, the frequency of Tregs that lost Foxp3 expression, so called ‘ex-Tregs’ [Ly5.2<sup>+</sup>Foxp3(RFP)<sup>-</sup>], was much higher in *Rag1*<sup>-/-</sup> mice engrafted with *cRel*<sup>-/-</sup> Tregs (Fig. 10B), indicating that c-Rel helps maintain Foxp3 expression. Collectively, these findings demonstrate that c-Rel maintains the immune suppressive properties and stability of peripheral Tregs.

## DISCUSSION

While previous studies showed that c-Rel is required for efficient production of pre-Tregs in the thymus, and additionally for efficient IL-2-mediated induction of Foxp3 in pre-Tregs [33, 37, 38], it has been unclear whether c-Rel is required for Treg TCR repertoire selection and whether c-Rel is required for post-thymic Treg differentiation and function. Here we found that in mice lacking c-Rel, the mature thymic Treg TCR repertoire was normal, although a reduction in splenic Treg TCR diversity was observed. However, c-Rel-deficient thymic pre-Treg exhibited subtle but reproducible abnormalities in TCR signalling and TCR repertoire. We found that c-Rel also contributes to the transcriptional program in newly generated thymic Tregs and likewise in peripheral Tregs, where in the later c-Rel promotes cellular division, Treg stability, the size of tissue-resident Treg populations and effector activity.

Whether the impaired generation of *cRel*<sup>-/-</sup> pre-Tregs in response to negative selection reflects a failure to promote a distinct developmental program required by most pre-Tregs or simply a cell survival defect, remains unclear [30]. Here we show that, despite nuclear c-Rel being present in pre-Tregs, the gene expression pattern in remaining *cRel*<sup>-/-</sup> pre-Tregs is largely unchanged compared to the *cRel*<sup>+/+</sup> control, thereby establishing that c-Rel is largely dispensable for the generation and maintenance of a unique transcriptional programme in these cells. This in turn supports a model in which c-Rel drives pre-Treg development by promoting cell survival [74, 75]. This is indeed supported by the observation that *cRel*<sup>-/-</sup> CD4SP thymocytes that fail Treg differentiation, undergo normal clonal deletion in the 3A9/insHEL model. Intact clonal deletion coupled with a normal Treg TCR repertoire suggest that c-Rel deficiency does not alter the TCR self-reactivity thresholds that control thymic Treg selection. While it is normal for many cells that attempt Treg differentiation to undergo clonal deletion

[76, 77], the numerical pre-Treg deficiency in *cRel*<sup>-/-</sup> mice suggests that an even greater proportion of such cells undergoes clonal deletion when c-Rel is absent. The fates of nascent CD4SP thymocytes attempting Treg differentiation in wild-type and gene-deficient mice demonstrate that the clonal deletion that is prevented by Card11/NF-κB occurs earlier than the clonal deletion that is prevented by IL-2 signalling [77]. Together, these data would imply that the pre-Treg population in *cRel*<sup>-/-</sup> mice is more likely to consist of developmentally “younger” cells that have yet to undergo clonal deletion, than the *cRel*<sup>+/+</sup> pre-Treg population which are more likely to survive this checkpoint.

The notion that the pre-Treg populations in *cRel*<sup>+/+</sup> and *cRel*<sup>-/-</sup> mice differ in the period of post-antigen recognition provides an explanation for several results obtained here (Fig. Sup. 10). As Nur77(GFP) expression increases for at least 12 hours after the onset of strong TCR signalling [48], the lower Nur77(GFP) expression in *cRel*<sup>-/-</sup> pre-Treg may arise from an overrepresentation of immature pre-Tregs in *cRel*<sup>-/-</sup> mice. Both the hydrophobic index and TCR overlap analyses showed that in *cRel*<sup>+/+</sup> mice pre-Tregs were more similar to thymic Tregs, which is in contrast to *cRel*<sup>-/-</sup> pre-Tregs that differ from *cRel*<sup>-/-</sup> Tregs. If these effects arise from the putative difference in pre-Treg maturity, then it would imply that clonal deletion progressively eliminates unfit thymocytes during the pre-Treg stage, which lasts > 48 hours in mice [78], and that c-Rel is required for the survival of most thymocytes undergoing this phase of pre-Treg maturation. In CD4<sup>+</sup> thymocytes undergoing TCR signalling above the threshold that induces tolerance mechanisms, cells with high [79, 80] or low [79, 81] affinity or avidity for self-antigen usually undergo clonal deletion, whereas those in an intermediate range are more efficient at completing Treg differentiation. Despite the abnormalities we detected at the pre-Treg stage, the TCR-dependent selection of thymocytes in the intermediate range that

progress to the Foxp3<sup>+</sup> stage appear to be unaffected by the absence of c-Rel, based on the normal Nur77(GFP) and TCR repertoire of thymic Treg in *cRel*<sup>-/-</sup> mice.

Within pre-Tregs, the IL-2 induction of Foxp3 is partially c-Rel-dependent [37, 38], although how c-Rel and cytokine signalling were linked was unclear. We demonstrate that expression of GITR, OX40 and TNFR1, TNFRSF members that augment IL-2 induction of Foxp3 in pre-Tregs expressing lower affinity TCRs [54], are reduced on *cRel*<sup>-/-</sup> pre-Tregs, and that in the absence of c-Rel GITR ligand fails to enhance the IL-2-dependent induction of Foxp3 in culture. Based on these findings, we propose that a positive regulatory loop operates during Treg development, whereby c-Rel promotes the survival of pre-Treg and TNFRSF expression in pre-Tregs, thereby enhancing c-Rel-dependent *Foxp3* transcription in response to TNFRSF ligand co-stimulation by thymic APCs. This agrees with the findings of Zhan *et al.* (2008), in which canonical NF-κB proteins were found to be important for GITR-mediated survival in conventional T cells following weak TCR stimulation, and subsequently for transducing signals downstream of GITR ligation [82].

In addition to enhancing the induction of Foxp3 expression during Treg development, c-Rel was found to control an extensive transcriptional programme in newly formed CCR7<sup>+</sup> thymic Tregs that encompasses over 700 genes. Similar numbers of genes were upregulated or down-regulated in the absence of c-Rel, consistent with c-Rel being both a positive and negative regulator of transcription [83]. However, given 4 to 5% of the altered genes were transcription factors, a key aim of future studies will be to determine which of these genes are direct c-Rel targets versus indirect targets impacted by c-Rel regulated transcription factors. Indeed, amongst the transcription factors impacted by the loss of c-Rel is a group previously shown to act in synergy with Foxp3 to promote the expression of Treg signature genes and to enhance

the Foxp3 occupancy of genomic targets [23]. To what extent altered expression of each of these individual key transcriptional regulators change the pattern of gene expression in *cRel*<sup>-/-</sup> thymic Treg remains to be determined. Pathway analysis indicates that T cell activation and differentiation are the main functions impacted by c-Rel during Treg development, a finding consistent with the c-Rel being intimately associated with numerous aspects of immune cell activation [84]. While the signals driving c-Rel activation during the pre-Treg to Treg transition that are responsible for promoting the totality of c-Rel-dependent gene expression remain to be formally established, the evidence we present here strongly implicates TNFRSF signalling. In agreement with this finding, GITR signalling has recently been implicated in controlling the constitutive activation of RelA in peripheral Tregs [45].

We also identified a dramatic reduction of CCR6<sup>+</sup>CCR7<sup>-</sup> mature Tregs in the thymi of *cRel*<sup>-/-</sup> *Foxp3*<sup>rfp</sup> mice compared to their wild-type counterparts. This absence correlates with a decrease of thymic effector-phenotype Treg cells, a phenotype associated with recirculating CCR6<sup>+</sup> Tregs [58]. Interestingly, a recent report identified CCR6<sup>+</sup> mature thymic Tregs as predominately expressing the decoy IL-1 receptor 2 (*Il1r2*), which fine-tune tTreg development by mopping up extraneous, inhibitory IL-1β in the thymus [85]. In addition to a lack of eTreg-phenotype cells in the thymi of *cRel*<sup>-/-</sup> *Foxp3*<sup>rfp</sup> mice, there is also a reduced expression of *Il1r2* mRNA in newly developed CCR7<sup>+</sup> thymic Tregs and splenic Nr1<sup>+</sup> tTregs. Whether the reduction in CCR6<sup>+</sup> mature Tregs in c-Rel-deficient thymi leads to a loss of IL-1β-sensitive Tregs, or conversely the lack of *Il1r2* in *cRel*<sup>-/-</sup> Tregs reduces the capacity of mature Tregs to recirculate to the thymus, cannot be established by these experiments; however, this suggests another possible point of regulation by c-Rel in Treg ontogeny.

Following tTreg thymic egress, our findings show that c-Rel activity continues to be required to control different aspects of Treg biology. While nuclear c-Rel levels were found to be minimal in cTregs as compared to eTregs, our data indicates that c-Rel still contributes to the homeostasis of cTregs. However, c-Rel has its greatest impact on eTregs, with eTreg numbers preferentially reduced in *cRel*<sup>-/-</sup> mice. The reduction in *cRel*<sup>-/-</sup> eTreg numbers does not appear to result from impaired eTreg differentiation. The frequency of *cRel*<sup>-/-</sup> eTregs expressing Blimp is similar to that of *cRel*<sup>+/+</sup> eTregs, with *cRel*<sup>-/-</sup> cTreg cells also able to up regulate activation markers characteristic of eTregs in response to TCR stimulation *in vivo*. Instead, impaired homeostatic proliferation appears to be responsible for the reduction in the eTreg population. Indeed, RNA-seq analysis of splenic Nr1<sup>+</sup> tTregs, which comprise a mixture of cTregs and eTregs, reveals that ~25% of changes in the *cRel*<sup>-/-</sup> Treg population correspond to a reduction in expression of genes encoding cell cycle proteins. Ki67 stains confirm that peripheral *cRel*<sup>-/-</sup> tTregs undergo less homeostatic proliferation, a finding that is in line with previous evidence showing that the division of eTregs is greater than that of cTregs [13, 86]. Notably, loss of Myb, another important regulator of eTreg differentiation and function, similarly results in impaired cell cycle progression and expression of TNFRSF proteins [13], pointing to cross-talk between these pathways in controlling Treg function and maintenance. This central characteristic of peripheral *cRel*<sup>-/-</sup> Tregs fits perfectly with the established role c-Rel plays in lymphocyte division [87-91].

The activation of c-Rel in peripheral tTregs is most likely driven by TCR signalling. The levels of Nur77(GFP) are low in *cRel*<sup>-/-</sup> tTregs from secondary lymphoid organs. Moreover, *cRel*<sup>-/-</sup> T cells have a reduced capacity to upregulate Foxp3 at low levels of TCR stimulation *in vitro*, partially due to an inability to produce IL-2 in response to weak stimulation. Collectively, these results suggest that c-Rel-deficient Tregs receive lower apparent TCR signals than their wild-

type counterparts. Previous studies show tonic TCR signalling in peripheral tTregs is essential for maintaining homeostatic proliferation of both eTregs and cTregs [92, 93]. Importantly in one of these reports examining the impact post-developmental TCR signalling has on peripheral Tregs identified *cRel* as one of the genes whose expression is significantly diminished in the absence of a mature TCR [92]. Whilst 89 of the differentially expressed genes are shared by *cRel*<sup>-/-</sup> thymic and peripheral tTregs, these genes are not involved in cell cycle regulation, but instead are associated with various aspects of immune function. This observation shows that c-Rel controls distinct peripheral Treg functions in part via a subset of genes enriched for pathways involved in immune cell function whose c-Rel-dependent pattern of expression appears to be established during tTreg development. There are four genes differentially expressed in pre-Tregs, newly derived thymic Tregs and splenic tTregs; *Lad1*, *Cd83*, *Colq* and *St8sia6*. *Lad1* is canonically a basement membrane protein that has nonetheless been identified in a proteomics screen as a non-TCR related, Treg specific protein [94]. *Cd83* has been identified as being important for eTreg homeostasis in a TCR-dependent manner [92], as well as being a c-Rel target [95, 96]. Separately, both *Lad1* and *St8sia6* were also identified in a list of the top 200 differentially expressed genes between cTregs and eTregs [71]. How these genes influence Treg function has yet to be established, however, their reduced expression through-out Treg ontogeny in *cRel*<sup>-/-</sup> mice raises the intriguing notion that that c-Rel control of eTreg homeostasis may be initiated by c-Rel in the thymus. By contrast, controlling peripheral Treg division appears to be a post-developmental feature of c-Rel function, which we propose is dependent on TCR signalling, in line with the previous findings of Rudensky and colleagues [92].

Consistent with c-Rel serving multiple functional roles in peripheral Tregs, cells lacking c-Rel were found to have a reduced capacity to restrain conventional T cell proliferation in a



lymphopenic environment. *cRel*<sup>-/-</sup> Treg numbers were markedly lower and the frequency of Foxp3<sup>+</sup> ex-Tregs was dramatically increased, indicating Treg stability is reduced in the absence of c-Rel, a finding reminiscent of the absence of RelA [39]. Notably, c-Rel-deficient Tregs were actually capable of controlling lymphopenic T cell proliferation, as well as maintaining Treg stability in the mesenteric lymph nodes, highlighting the impact that location has on c-Rel control of Treg function. Two recent reports from Ghosh and colleagues demonstrate that c-Rel was crucial for the generation and maintenance of eTregs [38, 96]. The importance of c-Rel in eTreg function was reinforced by showing tumour growth was reduced in mice with c-Rel-deficient Tregs [96]. While the broad conclusions on c-Rel function in peripheral Tregs reached by Grinberg-Bleyer *et al.* are supported by our findings, including eTreg maintenance, we did not find that c-Rel was needed for eTreg generation *per se*. Instead, our data indicates the marked reduction in *cRel*<sup>-/-</sup> eTregs is likely due to impaired homeostatic proliferation. This difference may reflect the use of different mouse models in the two studies; our study in which c-Rel is absent throughout T cell development and differentiation, versus Grinberg-Bleyer *et al.*, who used the *Foxp3*<sup>cre</sup> deleter model to explore the function of c-Rel following Foxp3 expression; although the frequency of Ki67<sup>+</sup> Tregs was also found to be lower in *cRel*<sup>fl/fl</sup>*Foxp3*<sup>cre</sup> mice in agreement with our analyses [97]. With c-Rel activity during development shown here to contribute uniquely to the transcriptional landscape in both developing and peripheral Tregs, it is likely that the loss of c-Rel at different junctures in development and differentiation will result in distinct functional outcomes. Dissecting the c-Rel-mediated instructions set up during thymic development from additional c-Rel-dependent signals received in the periphery, which collectively direct the function of mature Tregs, will be important for understanding the distinct roles c-Rel plays during a Treg's life span.

In summary, we establish that c-Rel serves as a key transcriptional regulator that bridges Treg development and function, employing different mechanisms regulated by distinct signals that impinge on a range of Treg physiological functions that include cell survival, cellular activation, cell division and immune function. Importantly, we also show that c-Rel contributes to the peripheral Treg transcriptional landscape at both the developmental and post-developmental stages of Treg ontogeny. The finding that c-Rel serves as a regulatory T cell checkpoint in controlling cancer growth [96], coupled with the finding that in mice inhibiting c-Rel ameliorates autoimmune diseases including arthritis [98] and SLE [99] with little, if any, adverse impact reinforces the importance of developing and employing c-Rel modulatory drugs in the clinical setting.

## Materials and Methods

### *Mice*

The following mouse strains were maintained on an inbred Ly5.2 C57BL6/J background (>10 generation backcross). Unless otherwise stated, mice were age and sex matched within experiments. *cRel*<sup>-/-</sup> [87] and *Foxp3*<sup>rfp</sup> mice [100] have been described previously. The *Foxp3*<sup>rfp</sup> and C57BL6/J Ly5.1 strains were inter-crossed to create Ly5.1*Foxp3*<sup>rfp</sup> mice. *Lck*<sup>cre</sup>*Rel*<sup>wt/flox</sup> mice heterozygous for a loxP targeted c-Rel allele [55], and carry a Cre transgene expressed under the control of the Lck promoter (*Lck*<sup>cre</sup>) were inter-crossed with *Foxp3*<sup>rfp</sup> mice to generate *Lck*<sup>cre</sup>*Rel*<sup>wt/flox</sup>*Foxp3*<sup>rfp</sup> offspring. These mice were then used to generate litter-matched mice homozygous for the wild-type (*Lck*<sup>cre</sup>*Rel*<sup>wt/wt</sup>*Foxp3*<sup>rfp</sup>) or floxed (*Lck*<sup>cre</sup>*Rel*<sup>fl/fl</sup>*Foxp3*<sup>rfp</sup>) c-Rel genes. Nur77<sup>gfp</sup> [48], Blimp<sup>gfp</sup> [70] or Yae62β-tg [49] mice were interbred with *cRel*<sup>-/-</sup> mice carrying the *Foxp3*<sup>rfp</sup> allele to give *cRel*<sup>+/-</sup>*Foxp3*<sup>rfp</sup>*Nur77*<sup>gfp</sup>, *cRel*<sup>+/-</sup>*Foxp3*<sup>rfp</sup>*Prdm1*<sup>gfp</sup> and *cRel*<sup>+/-</sup>*Foxp3*<sup>rfp</sup>*Yae62β*<sup>tg</sup> mice respectively. *cRel*<sup>-/-</sup> mice were backcrossed to 3A9 TCR-transgenic mice [101] on the B10.BR (*H2*<sup>k</sup>) background for 3 generations then intercrossed to produce mice used as bone marrow donors. Bone marrow chimeras were generated by irradiating recipient Ly5.1 (CD45.1) mice on the B10.BR background, some of which carried the insHEL transgene [102] with x-rays (two doses of 4.5 Gy given 4 hr apart) and injecting at least 2x10<sup>6</sup> bone marrow cells intravenously on the same day.

The genotypes of the different mouse strains were determined by PCR screening of tail or ear clip samples; specific PCR protocols are available upon request. *Rag1*<sup>-/-</sup> mice were bred at Monash Animal Research Platform (MARF) or purchased from the Walter and Eliza Hall Institute (WEHI). The experiments outlined in this study used age and sex-matched mice bred in specific pathogen free facilities at MARF (Monash University, Clayton) or the Australian

Phenomics Facility, Canberra. All mouse experiments were performed in accordance with the animal ethics guidelines of the National Health and Medical Research Council of Australia with the approval of the Alfred Medical Research and Education Precinct and MARP or Australian National University animal ethics committees.

### *Reagents*

Antibodies specific for mouse c-Rel, RelA and NF- $\kappa$ B1 used for electrophoretic mobility supershift assays were purchased from Santa Cruz Biotechnology. The antibodies used for flow cytometry were purchased from BD Biosciences, eBiosciences and Biolegend (see Sup. Table 1 for details) or hybridoma supernatant containing the anti-TCR<sup>3A9</sup> 1G12 monoclonal antibody (mouse IgG1,  $\kappa$ ) was used. Live/Dead Fixable Aqua Dead Cell Stain Kits were purchased from Invitrogen Life Technologies. Recombinant GITR ligand and OX40 ligand were purchased from Novus Biologicals, recombinant mouse IL-2 from eBioscience and TGF- $\beta$  from RnD systems. LEAF purified anti-CD3 (145-2C11) and anti-CD28 (37N51) antibodies were used for *in vitro* stimulation of T cells (WEHI hybridoma facility).

### *Lymphocyte isolation*

Thymus, spleen and lymph node cells were isolated and prepared as previously described [39]. Lymphocytes in the lung and small intestine were isolated by enzymatic digestion followed by density gradient enrichment. Briefly, solid organs diced in 5 mL of Hanks Ca<sup>2+</sup> and Mg<sup>2+</sup> free medium containing 2% FCS were incubated for 20-30 min with gentle agitation at 37°C in RPMI containing DNase, collagenase and dispase. The digested tissue was pushed through a 70  $\mu$ m filter and the resulting cell suspension pelleted by centrifugation. Cells were resuspended in 2 mL of HBSS, then under-laid with 2 mL of Histopaque 1077 (Sigma-Aldrich) and centrifuged at 2200 rpm for 20 min at 25°C. VAT diced in 5 mL of Dulbeccos's

PBS was incubated for 1 h with gentle agitation at 37°C in DPBS containing collagenase IV. The digested tissue was pushed through a 70 µm filter and the resulting cell suspension pelleted by centrifugation. Cells were washed in complete media, then resuspended in 30% Percoll (Sigma-Aldrich) and centrifuged at 1200 rpm for 10 min with the break off. Lymphocytes were collected from the interface and washed in HBSS prior to performing antibody stains.

#### *Flow cytometry and cell sorting*

Flow cytometry and cell sorting were performed as per the *Guidelines for the use of flow cytometry and cell sorting in immunological studies* [103]. Single cell suspensions from thymus, spleen, peripheral lymph nodes (pLNs), liver, small intestine, VAT or lung stained with various antibody combinations and Live/Dead Aqua (Invitrogen Life Technologies) were acquired on an LSRII or LSR Fortessa X20 (BD Bioscience) flow cytometer and analysed using FlowJo software (TreeStar), or sorted using Influx or FACSARIA instruments (BD Bioscience). Where necessary, after completing stains for extracellular membrane bound proteins, cells were fixed and permeabilized using an anti-mouse/rat FOXP3 Staining Set (eBiosciences), then stained as per the manufacturer's instructions. Representative gating strategy for flow cytometry is outlined in Fig. Sup. 1A and B. Where required for the large-scale purification of CD4<sup>+</sup> T cell subsets using fluorescence activated cell sorting (FACS), samples were pre-enriched for CD4<sup>+</sup> T cells using MagniSort Mouse CD4 T cell Enrichment Kit, or a customised CD8<sup>+</sup> thymocyte depleting kit (eBioscience).

#### *Electrophoretic mobility shift assays (EMSA)*

Nuclear extracts were generated, and electrophoretic mobility shift assays performed using FACS sorted conventional T cell [CD4<sup>+</sup>CD25<sup>hi</sup>GITR<sup>+</sup>Foxp3(RFP)<sup>-</sup>] and *de novo* Treg [CD4<sup>+</sup>CD25<sup>hi</sup>GITR<sup>+</sup>Foxp3(RFP)<sup>+</sup>CCR7<sup>+</sup>] populations, essentially as described [104]. Briefly,

equivalent amounts of nuclear extracts incubated with a  $^{32}\text{P}$ dATP (Amersham) labelled  $\kappa\text{B3}$  probe from the mouse c-Rel promoter were fractionated on non-denaturing polyacrylamide gels, dried and subjected to autoradiography. For antibody super-shift experiments, nuclear extracts were initially incubated on ice for 30 min with antibodies specific for mouse c-Rel (Santa Cruz Biotechnology) prior to adding radiolabelled probe.

#### *T cell cultures*

Purified  $\text{CD4}^+$  T cells were cultured in Gibco SensiCell RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% heat inactivated foetal calf serum (FCS), 50 mM  $\beta$ -mercaptoethanol, penicillin (100 mg/mL), streptomycin (5 mg/mL) and 10 mM HEPES in a humidified 10%  $\text{CO}_2$  atmosphere at  $37^\circ\text{C}$ .

Five-thousand thymic pre-Tregs [ $\text{CD4}^+\text{SPCD25}^{\text{hi}}\text{Foxp3(RFP)}^-$ ] were isolated from  $c\text{Rel}^{+/+}$  or  $c\text{Rel}^{-/-}$  mice, stained with CellTrace Violet (Life Technologies) and incubated with various concentrations of IL-2 in the presence or absence of 50 nM GITR ligand or OX40 ligand in a 96-well flat-bottom plate. Twenty-four hours later cells were analysed for the induction of Foxp3 by RFP expression.

Naïve conventional T cells [ $\text{CD4}^+\text{CD25}^{\text{lo}}\text{CD44}^{\text{lo}}\text{Foxp3(RFP)}^-$ ] were isolated from  $c\text{Rel}^{+/+}$  or  $c\text{Rel}^{-/-}$  mice, stained with CellTrace Violet and cultured in a 96-well flat-bottom plate pre-coated with rat anti-mouse CD3 antibodies ranging from 0  $\mu\text{g/mL}$  to 5  $\mu\text{g/mL}$  and rat anti-mouse CD28 antibodies at a final concentration of 2  $\mu\text{g/mL}$ , and 2 ng/mL TGF- $\beta$ . Seventy-two hours later cells were analysed for the iTreg conversion by Foxp3(RFP) expression.

cTregs isolated from the pLN of *cRel*<sup>+/+</sup> or *cRel*<sup>-/-</sup> mice FACS sorted on the basis of CD4<sup>+</sup>CD25<sup>hi</sup>Foxp3(RFP)<sup>+</sup>CD44<sup>lo</sup> expression were cultured in a 96-well flat-bottom plate pre-coated with rat anti-mouse CD3 antibodies ranging from 0 µg/mL to 2.5 µg/mL and rat anti-mouse CD28 antibodies at a final concentration of 2 µg/mL for 72 hr. Expression of ICOS and CD44 were analysed by flow cytometry as a measure of the capacity of *cRel*<sup>-/-</sup> cTregs to differentiate into eTregs.

Conventional Foxp3(RFP)<sup>-</sup> CD4<sup>+</sup> T cells were isolated from the spleen of *cRel*<sup>+/+</sup>*Foxp3*<sup>rfp</sup>*Nur77*<sup>gfp</sup> or *cRel*<sup>-/-</sup>*Foxp3*<sup>rfp</sup>*Nur77*<sup>gfp</sup> mice and cultured on a 96-well flat-bottom plate pre-coated with rat anti-mouse CD3 antibodies ranging from 0 µg/mL to 2.5 µg/mL and rat anti-mouse CD28 antibodies at a final concentration of 2 µg/mL for 8 hr. Expression of Nur77(GFP) was analysed by flow cytometry as a measure of the capacity of *cRel*<sup>-/-</sup> cTregs to differentiate into eTregs.

#### *In vivo T cell suppression assays*

Tregs [CD4<sup>+</sup>CD25<sup>hi</sup>Foxp3(RFP)<sup>+</sup>] were isolated from the pooled spleens and pLNs of *cRel*<sup>+/+</sup>*Foxp3*<sup>rfp</sup> or *cRel*<sup>-/-</sup>*Foxp3*<sup>rfp</sup> mice, while CD4<sup>+</sup> Tcon [CD4<sup>+</sup>CD25<sup>-</sup>Foxp3(RFP)<sup>-</sup>] were prepared from the spleen and pLNs of Ly5.1*Foxp3*<sup>rfp</sup> mice. Following the enrichment of CD4<sup>+</sup> cells using a MagniSort CD4<sup>+</sup> T cell kit (eBioscience), Tregs and CD4<sup>+</sup> Tcon were purified by flow cytometry (> 98% final purity). 3 x 10<sup>5</sup> Ly5.1<sup>+</sup>CD4<sup>+</sup>Foxp3(RFP)<sup>-</sup> Tcon alone, or with 1 x 10<sup>5</sup> Ly5.2<sup>+</sup> *cRel*<sup>+/+</sup> or *cRel*<sup>-/-</sup> Treg cells were intravenously injected into *Rag1*<sup>-/-</sup> mice. Four weeks later, cell suspensions were prepared from the spleen, pLNs and mLNs of individual mice and analysed by flow cytometry.

#### *RNA-seq analysis*

Purified pre-Treg [CD4<sup>+</sup>CD25<sup>hi</sup>GITR<sup>+</sup>Foxp3(RFP)<sup>-</sup>] and Treg [CD4<sup>+</sup>CD25<sup>hi</sup>GITR<sup>+</sup>CCR7<sup>+</sup>Foxp3(RFP)<sup>+</sup>] populations were isolated from the thymi or spleen of *cRel*<sup>+/+</sup>*Foxp3*<sup>rfp</sup> and *cRel*<sup>-/-</sup>*Foxp3*<sup>rfp</sup> mice using MagniSort CD4<sup>+</sup> T cell enrichment followed by FACS sorting. Total RNA was extracted from ~50 x 10<sup>3</sup> purified cells using a QIA Shredder spin column and followed by the RNeasy Micro Kit (Qiagen) protocol. The quality and quantity of the RNA samples was assessed using an Agilent Bioanalyzer (Agilent Technologies), with all samples used for RNA sequencing analysis having a RIN of > 8. The library preparation and RNA sequencing of Treg RNA samples was performed at Micromon (Monash University, Clayton) using an Illumina NextSeq500 machine. Image and bioinformatic analysis were performed at the Monash University Bioinformatic Platform. The sequencing data was processed using the RNA-sik pipeline [105] against the *Mus musculus* reference GRCm38. This includes mapping the reads using the STAR [106] aligner, and quantifying against the GRCm38.84 annotation with feature Counts [107]. Statistical testing for differential gene expression was performed using Degust [108], with a cut-off of FDR < 0.05. Ingenuity Pathway Analysis (Qiagen) was performed on differentially expressed genes.

#### *TCR sequencing and filtering*

Methods used for RNA isolation, cDNA synthesis, PCR amplification of TCR $\alpha$  and TCR $\beta$  transcripts, addition of sequencing adapters and sample indices, amplicon concentration, purification, sequencing and alignment to mouse genome using molecular identifier groups-based error correction (MIGEC) software [109] were described previously [51]. Primers are listed in Supplementary Table 2. Sequences with a CDR3 that was out-of-frame or contained a stop codon were excluded. To avoid overestimating TCR diversity due to PCR or sequencing errors, sequences detected only once in any given sample were excluded. A clonotype was defined as a unique combination of V gene and CDR3 amino acid sequence. Each clonotype



was counted only once per sample. Due to duplication events at the mouse TCR $\alpha\delta$  locus, some reads aligned to > 1 *Trav* paralog; these reads were assumed to use the *Trav* paralog listed first by MIGEC.

### *TCR sequence analyses*

For hydrophobic index calculation, CDR3 sequences < 8 amino acids were excluded because a conserved Phe or Try is present at position 6 or 7 of CDR3 sequences that are 6 or 7 amino acids long. The hydrophobic index equals the percentage of clonotypes with a CDR3 position 6-7 doublet corresponding to any of the 175 amino acid doublets identified as promoting T-cell self-reactivity [50]. *Trav–Traj* diversity and Morisita Horn index calculations were performed at the level of TCR catalogue, defined as the aggregate of all clonotypes detected in samples of a given combination of mouse genotype and T-cell subset. Clonotype abundance in a TCR catalogue equals the number of mice in which the clonotype was detected. The number of clones in a TCR catalogue is the sum of the abundances of all clonotypes. The iNEXT software package was used to produce sample size-based and coverage-based rarefaction and extrapolation curves, with 95% confidence bands based on 5 bootstrap replications [53]. Relative diversity estimates were performed following the approach of Chao and Jost [52], whose term “individual” we equate with “clone”, and “species” we equate with “*Trav–Traj* combination”. Sample coverage is defined as the proportion of all clones predicted to exist in a TCR catalog (including undetected ones) that use a *Trav–Traj* combination detected in the TCR catalogue. As *Trav–Traj* combination diversity was calculated using the diversity order (Hill number or  $q$ ) of 1, Shannon entropy is equal to the natural log of the number of unique *Trav–Traj* combinations [110]. Morisita Horn indices were computed using EstimateS (Version 9.1.0, R. K. Colwell, <http://purl.oclc.org/estimates>).

### *Statistical analyses*

The “tidyverse”, “stringr”, “reshape2” and “iNEXT” packages were used in RStudio software for TCR sequence analyses and to produce graphs. Other statistical analyses were performed using GraphPad Prism versions 7.0a or 8.4.2 (GraphPad Software, La Jolla California USA) with multiple comparison tests recommended in the GraphPad Prism software. Graphs are presented as mean  $\pm$  SEM. Venn diagrams were produced using Venny 2.1 (J. C. Oliveros, <https://bioinfogp.cnb.csic.es/tools/venny/index.html>). Some figures were made using Adobe Illustrator CC (Adobe Systems Inc., San Jose, CA).

### *Data Availability*

RNA-seq data has been deposited in the GEO databank and is accessible under the accession number GSE154166.

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## **Conflicts of Interest**

The authors declare that they have no commercial or financial conflict of interest.

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## Figure Legends

**Figure 1. c-Rel is constitutively active in the nucleus of developing Tregs.** A) The frequency of thymic Treg populations were examined in *cRel*<sup>+/+</sup>*Foxp3*<sup>flp</sup> and *cRel*<sup>-/-</sup>*Foxp3*<sup>flp</sup> mice by flow cytometry using the gating strategy outlined in Fig. Sup. 1A. \*\*\*  $p < 0.001$ , 2way ANOVA with Bonferroni's correction;  $n = 6$  mice from 4 independent experiments with 1 or 2 biological replicates per experiment. Data are presented as mean  $\pm$  SEM. B) Nuclear extracts were prepared from pre-Tregs ( $CD4^+CD25^{hi}GITR^{hi}Foxp3^-$ ) and newly developed ( $CD4^+CD25^{hi}Foxp3^+CCR7^+$ ) Tregs purified from the thymus of *cRel*<sup>+/+</sup>*Foxp3*<sup>flp</sup> mice by cell sorting and examined for binding to the  $\kappa B3$  probe by EMSA (Grumont & Gerondakis, 1994). Nuclear extracts prepared from pre-Tregs (C) and newly developed Tregs (D) isolated by cell sorting were pre-incubated with antibodies towards c-Rel before being examined for binding to the  $\kappa B3$  probe by EMSA. Major NF- $\kappa B$  complexes are indicated by arrows. The data is representative of 3 independent experiments.

**Figure 2. Certain abnormalities are detectable within *cRel*<sup>-/-</sup> pre-Treg, but not *cRel*<sup>-/-</sup> thymic Treg.** A) The level of Nur77 was measured by flow cytometry in developing *cRel*<sup>+/+</sup> or *cRel*<sup>-/-</sup> thymic Tregs using a Nur77(GFP) reporter. The representative histograms are shown on the left and the data are presented as mean  $\pm$  SEM on the right. \*\*\*  $p < 0.001$ , 2way ANOVA with Bonferroni's correction;  $n = 7$  mice from 4 independent experiments with 1 or 2 biological replicates per experiment. B and C) Pre-Treg or Treg cells were sorted from the thymi of *cRel*<sup>+/+</sup>, *cRel*<sup>+/-</sup> and *cRel*<sup>-/-</sup> Yae62 $\beta$ -tg mice, TCR $\alpha$  transcripts amplified by sequential PCR (primers listed in Sup. Table 2) and sequenced on an Illumina NextSeq machine. Sequences were aligned to V (D) and J gene segments, and CDR3 amino acid sequences were ascertained, using molecular identifier groups-based error correction (MIGEC) software. B)

Hydrophobic index equals the percentage of clonotypes with a CDR3 position 6-7 doublet corresponding to any of the 175 amino acid doublets identified as promoting T-cell self-reactivity. \*  $p < 0.05$ , 1way ANOVA with Tukey's multiple comparisons test;  $n = 4-7$  mice / group from 1 experiment. C) For the  $cRel^{+/+}$ ,  $cRel^{+/-}$  and  $cRel^{-/-}$  thymic Treg TCR catalogues, rarefaction/extrapolation curves show the number of unique *Trav*–*Traj* combinations as a function of the number of unique TCR $\alpha$  clones (left). Circles show the observed values and shaded areas show 95% confidence bands determined using 5 bootstrap replications. Rarefaction/extrapolation curves as a function of coverage (middle). Relative *Trav*–*Traj* combination diversities calculated at the level of coverage indicated by the vertical line in the middle graph with error bars showing 95% confidence bands;  $n = 4-7$  mice / group from 1 experiment. D) From a previously published dataset [51], all TCR $\alpha$  clonotypes were assigned a “Relative frequency” (y-axis) based on their abundance (see Methods) in the CD4<sup>+</sup> Foxp3<sup>+</sup> Tcon (green) and/or CD4<sup>+</sup> Foxp3<sup>+</sup> Treg (orange) catalogues aggregated from thymus and spleen of wild-type Yae62 $\beta$ -tg mice. Shown in white on each of the 6 graphs in the upper panel is the number of TCR $\alpha$  clonotypes also present in a given TCR catalogue from  $cRel^{+/+}$ ,  $cRel^{+/-}$  or  $cRel^{-/-}$  Yae62 $\beta$ -tg pre-Tregs or Tregs. Individual TCR $\alpha$  clonotypes were arranged left to right from Treg-biased to CD4<sup>+</sup> Tcon-biased. To enable statistical analyses, the area shaded green or orange in the upper graph was converted to a percentage for each sample, represented by a circle in the lower panel of 2 graphs. \*  $p < 0.05$ , \*\*\*\*  $p < 0.0001$ , 1way ANOVA with Tukey's multiple comparisons test;  $n = 4-7$  mice / group from 1 experiment. E) Pre-Tregs were sorted from the thymi of  $cRel^{+/+}Foxp3^{rfp}$  and  $cRel^{-/-}Foxp3^{rfp}$  mice, mRNA isolated and sequenced using an Illumina NextSeq500 machine. Transcriptional data was processed using the RNA-sik pipeline against the *Mus musculus* reference GRCm38. Genes differentially expressed (FDR < 0.05) between  $cRel^{+/+}$  and  $cRel^{-/-}$  pre-Tregs are presented in a heatmap.  $n =$

2 (*cRel*<sup>+/+</sup>) or 3 (*cRel*<sup>-/-</sup>), where each n is pooled from 10-30 mice, from 2 independent experiments. Data is presented as mean  $\pm$  SEM.

**Figure 3. Developing *cRel*<sup>-/-</sup> Tregs do not augment the IL-2-induction of Foxp3.** A) Expression of OX40, TNFR2 and GITR proteins on *cRel*<sup>-/-</sup> pre-Tregs relative to *cRel*<sup>+/+</sup> pre-Tregs by flow cytometry. The representative histograms are shown on the left and the data are presented as mean  $\pm$  SEM on the right. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , 2way ANOVA with Bonferroni's correction;  $n = 3$  (OX40, TNFR2) from 3 independent experiments or 7 (GITR) from 6 independent experiments with 1 or 2 biological replicates per experiment. B) CD4<sup>+</sup>CD25<sup>hi</sup>Foxp3(RFP)<sup>-</sup> pre-Tregs isolated from *cRel*<sup>+/+</sup>*Foxp3*<sup>rfp</sup> and *cRel*<sup>-/-</sup>*Foxp3*<sup>rfp</sup> mice were cultured with various concentrations of IL-2 alone (dotted line) or in the presence of GITRL (solid line) and examined for Foxp3(RFP) induction by flow cytometry. \*  $p < 0.05$ , \*\*\*  $p < 0.001$ , 2way ANOVA with Bonferroni's correction;  $n = 3$  from 3 independent experiments with 1 biological replicate per experiment. C) The frequency of developing pre-Tregs and Tregs examined in the thymi of *Lck*<sup>cre</sup>*cRel*<sup>wt/wt</sup>*Foxp3*<sup>rfp</sup> and *Lck*<sup>cre</sup>*cRel*<sup>fl/fl</sup>*Foxp3*<sup>rfp</sup> mice by flow cytometry. The representative dot plots and histograms are shown on the left and the data are presented as mean  $\pm$  SEM on the right. \*  $p < 0.05$ , \*\*\*  $p < 0.001$ , 2way ANOVA with Bonferroni's correction;  $n = 3$  (*cRel*<sup>wt/wt</sup>) or 4 (*cRel*<sup>fl/fl</sup>) mice from 4 independent experiments with 1 biological replicate per experiment. Data is presented as mean  $\pm$  SEM.

**Figure 4. *cRel*<sup>-/-</sup> Tregs have a reduced effector-like phenotype.** A) Thymi of 12-week, 3-month and 9-month-old *cRel*<sup>+/+</sup>*Foxp3*<sup>rfp</sup> and *cRel*<sup>-/-</sup>*Foxp3*<sup>rfp</sup> mice were examined for the frequency of mature CCR6<sup>+</sup> Tregs by flow cytometry. The representative dot plots and histograms are shown on the left and the data are presented as mean  $\pm$  SEM on the right. \*\*\*  $p < 0.001$ , 2way ANOVA with Bonferroni's correction.  $n = 6$  mice (12 weeks and 6 months)

each from 2 independent experiments with 3 biological replicates per experiment or 3 mice (9 months) from 1 experiment with 3 biological replicates. B) Thymic Tregs were analysed by flow cytometry for expression of effector-like molecules. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , 2way ANOVA with Bonferroni's correction;  $n = 4$  (CD44<sup>hi</sup>CD62L<sup>lo</sup>), 5 (CD103), 6 (ICOS), 6 (TIGIT) mice each from 4 independent experiments with 1 or 2 biological replicates per experiment. C) Thymic Tregs from 12-week-old *Lck<sup>cre</sup>cRel<sup>wt/wt</sup>Foxp3<sup>rfp</sup>* and *Lck<sup>cre</sup>cRel<sup>fl/fl</sup>Foxp3<sup>rfp</sup>* mice were examined by flow cytometry for the frequency of *de novo* CCR7<sup>+</sup> and mature CCR6<sup>+</sup> Tregs. \*\*  $p < 0.01$ , 2way ANOVA with Bonferroni's correction.  $n = 3$  (*cRel<sup>wt/wt</sup>*) or 4 (*cRel<sup>fl/fl</sup>*) mice from 4 independent experiments with 1 biological replicate per experiment.

**Figure 5. c-Rel controls an extensive pattern of gene expression in *de novo* thymic Tregs.**

A) *De novo* CCR7<sup>+</sup> Tregs were sorted from the thymus of *cRel<sup>+/+</sup>Foxp3<sup>rfp</sup>* and *cRel<sup>-/-</sup>Foxp3<sup>rfp</sup>* mice, mRNA isolated and sequenced using an Illumina NextSeq500 machine. Transcriptional data was processed using the RNA-sik pipeline against the *Mus musculus* reference GRCm38. Heatmap of genes differentially expressed (FDR < 0.05) in newly derived thymic *cRel<sup>+/+</sup>* and *cRel<sup>-/-</sup>* Tregs.  $n = 2$ , where each  $n$  is pooled from 10-30 mice, from 2 independent experiments. B) Overlap of genes differentially expressed between pre-Tregs and thymic *de novo* Tregs in the absence of c-Rel. C) Top 20 canonical Ingenuity pathways impacted by the lack of c-Rel in thymic *de novo* Tregs. RA; Rheumatoid Arthritis. D) Heatmap of genes responsible for the Treg gene signature [12, 23, 59] differentially expressed (FDR < 0.05) in newly derived thymic *cRel<sup>+/+</sup>* and *cRel<sup>-/-</sup>* Tregs.  $n = 2$ , where each  $n$  is pooled from 10-30 mice, from 1 experiment.

**Figure 6. pTreg and iTreg generation is regulated by c-Rel.**

A) The frequency and number of Nrp1<sup>+</sup> pTregs were enumerated in peripheral tissues of *cRel<sup>+/+</sup>Foxp3<sup>rfp</sup>* and *cRel<sup>-/-</sup>Foxp3<sup>rfp</sup>*

mice by flow cytometry. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , 2way ANOVA with Bonferroni's correction;  $n = 8$  (spleen and pLN) from 8 independent experiments with 1 biological replicate and 6 (liver) from 2 independent experiments with 3 biological replicates. Naïve  $CD4^+CD44^{lo}Foxp3(RFP)^-$  T cells from  $cRel^{+/+}Foxp3^{rfp}$  and  $cRel^{-/-}Foxp3^{rfp}$  mice were cultured with varying concentrations of anti-CD3 antibodies, fixed concentrations of anti-CD28 antibodies and TGF- $\beta$ , then the B) frequency and number, C) proliferation and D) viability of  $Foxp3(RFP)^+$  iTregs was examined by flow cytometry. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ;  $n = 5$  mice from 5 independent experiments with 1 biological replicate. E) Naïve  $CD4^+CD44^{lo}Foxp3(RFP)^-$  T cells from  $cRel^{+/+}Foxp3^{rfp}$  and  $cRel^{-/-}Foxp3^{rfp}$  mice were cultured with varying concentrations of anti-CD3 antibodies, fixed concentrations of anti-CD28 antibodies and TGF- $\beta$  in the absence (dotted lines) or presence (solid lines) of IL-2, and the frequency of  $Foxp3(RFP)^+$  iTregs analysed by flow cytometry.  $n = 2$  from 2 independent experiments with 1 biological replicate. Data is presented as mean  $\pm$  SEM.

**Figure 7.  $cRel^{-/-}$  mice have the altered frequencies of eTregs in peripheral organs.** A) The frequency of  $CD44^{hi}CD62L^{lo}$ ,  $CD103^+$ ,  $ICOS^+$  and  $TIGIT^+$  cells amongst Tregs were examined by flow cytometry in the spleen and pLN from  $cRel^{+/+}Foxp3^{rfp}$  and  $cRel^{-/-}Foxp3^{rfp}$  mice. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , 2way ANOVA with Bonferroni's correction;  $n = 4$  mice each from 4 independent experiments with 1 biological replicate. B) The frequency of Tregs in the lymphocyte population was examined by flow cytometry in the visceral adipose tissue (VAT), small intestine (SI), lung and liver from  $cRel^{+/+}Foxp3^{rfp}$  and  $cRel^{-/-}Foxp3^{rfp}$  mice. \*\*  $p < 0.01$ , Mann-Whitney t-test;  $n = 6$  mice from 2 independent experiments with 3 biological replicates. C) Nuclear extracts of  $ICOS^{lo}$  cTregs or  $ICOS^{hi}$  eTregs isolated from the spleen of  $cRel^{+/+}Foxp3^{rfp}$  mice by cell sorting and probed for binding to  $\kappa B3$  by EMSA. Data shown is representative of 2 independent experiments. D) Expression of Blimp(GFP) was analysed by

flow cytometry in tTregs from *cRel*<sup>+/+</sup>*Foxp3*<sup>rfp</sup>*Prdm1*<sup>+/gfp</sup> and *cRel*<sup>-/-</sup>*Foxp3*<sup>rfp</sup>*Prdm1*<sup>+/gfp</sup> mice. The representative histograms are show on the left and the data are presented as mean  $\pm$  SEM on the right. GFP expression was compared to *cRel*<sup>+/+</sup>*Foxp3*<sup>rfp</sup>*Prdm1*<sup>+/+</sup> controls. Mann-Whitney t-test; n = 6 mice from 2 independent experiments with 3 biological replicates. Data is presented as mean  $\pm$  SEM.

**Figure 8. The lack of c-Rel impairs eTreg proliferation.** A) Nr1<sup>+</sup> tTregs were sorted from the spleen of *cRel*<sup>+/+</sup>*Foxp3*<sup>rfp</sup> and *cRel*<sup>-/-</sup>*Foxp3*<sup>rfp</sup> mice, mRNA isolated and sequenced using an Illumina NextSeq500 machine. Transcriptional data was processed using the RNA-sik pipeline against the *Mus musculus* reference GRCm38. Heatmap of genes differentially expressed (FDR < 0.05) in splenic *cRel*<sup>+/+</sup> and *cRel*<sup>-/-</sup> tTregs. n = 2 where each n is pooled from 5-10 mice, from 1 experiment. B) Overlap of genes differentially expressed between splenic *cRel*<sup>+/+</sup> and *cRel*<sup>-/-</sup> tTregs and the Blimp<sup>+</sup> eTreg signature identified by Vasanthakumar *et al.*, 2015. C) Top 20 canonical Ingenuity pathways impacted by the lack of c-Rel in splenic tTregs. D) The frequency of Ki67<sup>+</sup> tTregs was analysed by flow cytometry in *cRel*<sup>+/+</sup>*Foxp3*<sup>rfp</sup> and *cRel*<sup>-/-</sup>*Foxp3*<sup>rfp</sup> mice. \* p < 0.05, Mann-Whitney t-test; n = 4 mice from 3 independent experiments with 1 or 2 biological replicates. RA; Rheumatoid Arthritis. Data is presented as mean  $\pm$  SEM.

**Figure 9. c-Rel is important for maintaining peripheral tTreg high affinity TCRs.** A) The level of Nur77 expression was measured by flow cytometry in splenic tTregs and pTregs from *cRel*<sup>+/+</sup>*Foxp3*<sup>rfp</sup> or *cRel*<sup>-/-</sup>*Foxp3*<sup>rfp</sup> mice using a Nur77(GFP) reporter. \* p < 0.05, multiple t-tests with Holm-Sidak correction; n = 5 mice from 4 independent experiments with 1 or 2 biological replicates. B) Hydrophobic index (see Methods) of the TCR $\alpha$  CDR3 repertoires of *cRel*<sup>+/+</sup>, *cRel*<sup>+/-</sup> and *cRel*<sup>-/-</sup> Yac62 $\beta$ -tg pre-Tregs or Tregs cells sorted from the thymus. n = 3-

4 mice / group from 1 experiment. C) The extent of TCR $\alpha$  clonotype overlap with a published dataset from CD4<sup>+</sup> Foxp3<sup>-</sup> Tcon (green) and/or CD4<sup>+</sup> Foxp3<sup>+</sup> Treg (orange) from wild-type Yae62 $\beta$ -tg mice was determined as described in Figure 2D. Shown in white on each of the graphs in the upper panel is the number of TCR $\alpha$  clonotypes also present in a given TCR catalogue from *cRel*<sup>+/+</sup>, *cRel*<sup>+/-</sup> or *cRel*<sup>-/-</sup> Yae62 $\beta$ -tg splenic Tregs. Individual TCR $\alpha$  clonotypes were arranged left to right from Treg-biased to CD4<sup>+</sup> Tcon-biased. To enable statistical analyses, the area shaded green or orange in the upper graph was converted to a percentage for each sample, represented by a circle in the lower panel of 2 graphs; n = 3-4 mice / group from 1 experiment. D) Rarefaction/extrapolation curves show the number of unique *Trav*-*Traj* combinations as a function of the number of TCR $\alpha$  clones (left) in *cRel*<sup>+/+</sup>, *cRel*<sup>+/-</sup> and *cRel*<sup>-/-</sup> splenic Tregs expressing the Yae62b Tg. Circles show the observed values and shaded areas show 95% confidence bands determined using 5 bootstrap replications. Rarefaction/extrapolation curves based on sample coverage, defined as the proportion of all TCR $\alpha$  clones predicted to exist that use a *Trav*-*Traj* combination present in the TCR catalogue (middle). Relative *Trav*-*Traj* combination diversities calculated at the level of coverage indicated by the vertical lines in (right) with error bars showing 95% confidence bands. n = 3 mice from 1 experiment.

**Figure 10. c-Rel-deficient Tregs have a reduced capacity to control peripheral proliferation in lymphopenic mice.** Foxp3(RFP)<sup>-</sup> conventional CD4<sup>+</sup> T cells isolated from C57BL/6J Ly5.1*Foxp3*<sup>rfp</sup> mice, and *cRel*<sup>+/+</sup> or *cRel*<sup>-/-</sup> Tregs from Ly5.2*Foxp3*<sup>rfp</sup> mice, were transferred to *Rag1*<sup>-/-</sup> hosts. Four weeks later the total number of Ly5.1<sup>+</sup>RFP<sup>-</sup> conventional T cells (A) from the spleen, pLN and mLN, and the percentage of ex-Tregs Ly5.2<sup>+</sup>Foxp3(RFP)<sup>-</sup> (B) were analysed by flow cytometry. \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001, 2way ANOVA



with Bonferroni's correction;  $n = 6$  mice from 4 independent experiments with 1 or 2 biological replicates. Data is presented as mean  $\pm$  SEM.

**Please add the supporting information (figures, tables and respective legends) as a separate single file**