BRG1-mediated immune tolerance: facilitation of Treg activation and partial independence of chromatin remodelling

Barbara H Chaiyachati, Anant Jani¹,
Yisong Wan^{1,2}, Haichang Huang,
Richard Flavell and Tian Chi*

Department of Immunobiology, Yale University Medical School,
New Haven, CT, USA

Treg activation in response to environmental cues is necessary for regulatory T cells (Tregs) to suppress inflammation, but little is known about the transcription mechanisms controlling Treg activation. We report that despite the known proinflammatory role of the chromatin-remodelling factor BRG1 in CD4 cells, deleting *Brg1* in all $\alpha\beta$ T cell lineages led to fatal inflammation, which reflected essential roles of BRG1 in Tregs. *Brg1* deletion impaired Treg activation, concomitant with the onset of the inflammation. Remarkably, as the inflammation progressed, Tregs became increasingly activated, but the activation levels could not catch up with the severity of inflammation. *In vitro* assays indicate that BRG1 regulates a subset of TCR target genes including multiple chemokine receptor genes. Finally, using a method that can create littermates bearing either a tissue-specific point mutation or deletion, we found the BRG1 ATPase activity partially dispensable for BRG1 function. Collectively, these data suggest that BRG1 acts in part via remodelling-independent functions to sensitize Tregs to inflammatory cues, thus allowing Tregs to promptly and effectively suppress autoimmunity.

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Introduction

FoxP3⁺ regulatory T cells (Tregs) are potent suppressors of inflammatory responses (Sakaguchi *et al*, 2008; Benoist and Mathis, 2012). Precise regulation of Treg activity is essential for preventing autoimmunity while simultaneously permitting adequate immune response to pathogens. Various Treg properties are subject to regulation, including activation, migration, homeostasis and functional specialization

(Campbell and Koch, 2011; Yamaguchi *et al*, 2011; Josefowicz *et al*, 2012). This regulation is first suggested by the observation that normal mice harbour both naïve-like and effector/memory-like Treg subsets, the former homing to secondary lymphoid organs while the latter express activation markers (such as CD69 and ICOS) and traffic to non-lymphoid tissues (Huehn *et al*, 2004). Tregs produced from the thymus are all naïve-like, but a subset of them quickly acquires the effector/memory-like phenotype after entering secondary lymphoid organs, which occurs as a result of encounters with self-antigens and is presumably essential for peripheral tolerance (Lee *et al*, 2007). Tregs are subject to an additional layer of regulation in the presence of overt inflammation (due to, e.g., pathogen infection or organ transplant), which can involve Treg activation, expansion, differentiation and trafficking (Belkaid *et al*, 2002; Suffia *et al*, 2006; Belkaid and Tarbell, 2009; Zhang *et al*, 2009). Treg activation is crucial for Treg function both *in vitro* and *in vivo* (Takahashi *et al*, 1998; Thornton and Shevach, 1998; Park *et al*, 2010).

The transcription programs controlling such intricate Treg properties are under intense investigation. The best-defined transcription factor in Tregs is FOXP3, which is required for Treg development, proliferation, suppressor function and lineage stability; loss-of-function mutations in *FoxP3* results in early-onset, aggressive, lethal inflammation in both humans and mice (Rudensky, 2011). FOXP3 acts by stabilizing and amplifying the expression of immunosuppressive genes while repressing proinflammatory genes. A number of other sequence-specific transcription factors have also been implicated in Treg development and function. These include the factors that promote *FoxP3* expression during development or homeostasis (such as FOXO, NF- κ B, GATA3 and FOXP3 itself) and those that direct Treg functional specialization in response to inflammatory cues (such as T-BET, IRF4 and STAT3 selectively required for Tregs to suppress Th1-, Th2- and Th17-type inflammation, respectively) (Chaudhry *et al*, 2009; Koch *et al*, 2009; Long *et al*, 2009; Zheng *et al*, 2009; Kerdiles *et al*, 2010; Ouyang *et al*, 2010; Zheng *et al*, 2010; Ouyang and Li, 2011; Wang *et al*, 2011). Much less is known regarding the transcription programme that controls Treg activation, even though Treg activation is essential for Treg function. Available data indicate that the activation mechanisms in Tregs are divergent from conventional CD4 cells. For example, contrary to conventional CD4 cells, Tregs do not proliferate upon TCR stimulation alone; IL-2 is additionally required (Campbell and Ziegler, 2007). Furthermore, while TCR stimulation of Tregs is obligatory for Treg-suppressive function *in vitro*, pharmacological inhibition of multiple signal transduction pathways known to be downstream of TCR in conventional CD4 cells fails to disable Tregs (Hagness *et al*, 2012). Furthermore, in conventional CD4 cells, NFAT is

*Corresponding author. Department of Immunobiology, Yale University Medical School, TCA640, 300 cedar street, New Haven, CT 06520, USA. Tel.: +1 203 785 7260; Fax: +1 203 785 4972; E-mail: Tian.Chi@yale.edu

¹These authors contributed equally to this work

²Present address: Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, NC, USA

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localized in the cytoplasm in resting cells and moves to the nucleus upon TCR stimulation, where it drives T-cell activation and proliferation. In contrast, in Tregs, a fraction of NFAT is constitutively nuclear, and calcium influx is dispensable for Treg proliferation induced by TCR/IL-2 stimulation (Li *et al*, 2012a). Finally, we found that resting Tregs expressed detectable amounts of *c-Fos* and *c-Jun* messages, which was unexpectedly downregulated within 2 h following TCR stimulation (unpublished).

To regulate gene expression, sequence-specific transcription factors must act in conjunction with enzymes that modulate chromatin structure. Such enzymes fall into two major classes: histone-modifying enzymes that covalently modify histones to alter chromatin structure, and ATP-dependent chromatin remodellers that use energy of ATP hydrolysis to physically disrupt histone–DNA contact, thus loosening or moving nucleosomes (Narlikar *et al*, 2002; Clapier and Cairns, 2009). The prototypical mammalian chromatin remodeler is the BAF chromatin-remodelling complex related to the yeast Swi/Snf complex (Wang, 2003; Chi, 2004). The BAF complex contains ~10 subunits, including the catalytic subunit BRG1. BRG1 is widely expressed, but its function is tissue-specific. For example, whereas BRG1 regulates the survival and CD4/CD8 expression in early thymocytes (Chi *et al*, 2002, 2003; Gebuhr *et al*, 2003; Jani *et al*, 2008), it promotes Th1/Th2 differentiation of conventional CD4 cells (Zhang and Boothby, 2006; Wurster and Pazin, 2008). Genome-wide mapping of BRG1-binding sites in conventional CD4 cells demonstrates that BRG1 often binds enhancer/promoters, and the binding patterns at some target genes vary according to the status of T-cell activation and/or effector lineage differentiation (De *et al*, 2011). Interestingly, using a genetic strategy that can produce littermates bearing either thymocyte-specific *Brg1* deletion or a BRG1 ATPase point mutation (PM) that abolishes its chromatin-remodelling activity, we found that the BRG1 PM cannot fully recapitulate the defects in early T-cell development caused by *Brg1* deletion. The data indicate that BRG1 harbours remodelling-independent activities sufficient for regulating the expression of some target genes in early thymocytes, but it is unclear whether such activities are of general importance (Jani *et al*, 2008). The role of BRG1 in Tregs is unknown. Here we report that BRG1 is essential for efficient Treg activation, and that BRG1 function is partially independent of its ATPase activity.

Results

Deletion of *Brg1* from all $\alpha\beta$ T-cell lineages results in late-onset inflammation in a fraction of mice

To study the role of *Brg1* in T cells, we generated *Brg1*^{F/F}; *CD4Cre* mice. PCR analysis confirmed that *Brg1* was deleted in DP and peripheral T cells (not shown). *Brg1* deletion did not grossly perturb T-cell development (Supplementary Figure S1) and young adults did not show any obvious phenotype. However, ~20% of the mice eventually developed overt inflammation (by ~5 months of age) characterized by rectal prolapse, colonic oedema, lymphadenopathy, splenomegaly and perivascular infiltration in multiple organs including liver and kidney (Figure 1A and B). In addition, in these mice, total lymphocyte count approximately doubled in peripheral lymph nodes (PLN) largely due to accumulation of

B cells (Figure 1C, column 1), while the abundance of CD4 relative to CD8 cells remained unaltered (Figure 1C, column 2). B cells also accumulated in the spleen, albeit to a less extent (Figure 1C, column 3). Finally, effector/memory CD4 cells accumulated in the periphery and CD4 cells overproduced IFN- γ but not IL-4 or IL-17 (Figure 1D). By ~7 months of age, these 20% of mice either died spontaneously or were euthanized for humane concerns. In contrast, the remaining 80% of the mice remained healthy.

Brg1 is not essential for Treg development or *in vitro* suppression

Given the known roles of *Brg1* in promoting Th1/Th2 differentiation, it was unexpected that *Brg1* deletion in T cells would lead to hyperinflammation rather than immune deficiency. We reasoned that since *Brg1* deletion in DP cells using the *CD4Cre* transgene depleted BRG1 not only in conventional T cells, which would impair immune responses, but also in Tregs, which might impair Treg development/function and enhance the immune responses, *Brg1* deletion in all T cells might have conflicting effects on inflammation, with the balance tipped towards hyperinflammation in some mice. Thus, we examined Tregs in *Brg1*^{F/F}; *CD4Cre* mice. Tregs were present in normal numbers and expressed normal levels of FOXP3, CD25, GITR and CTLA4 (Figure 1E and F), suggesting *Brg1* was dispensable for Treg development. To determine whether *Brg1* KO Tregs were functionally defective, we measured the ability of Tregs to inhibit the proliferation of CD4 cells *in vitro*. To avoid potential confounding effects of hyperinflammation on Treg function, *Brg1* KO Tregs were isolated from ~4-week-old *Brg1*^{F/F}; *CD4Cre* mice lacking overt inflammation. Tregs were co-cultured with naïve WT CD4 cells in the presence of irradiated antigen-presenting cells and anti-CD3, and the proliferation measured by H3-thymidine incorporation. As expected, Tregs did not proliferate while CD4 cells showed robust proliferation, incorporating high amounts (~5000 c.p.m.) of H3-thymidine, which was inhibited by WT Tregs in a dose-dependent manner (Figure 1G, white bars). Importantly, *Brg1* KO Tregs were even more effective than WT Tregs in suppressing CD4 cell proliferation in this assay (Figure 1G, black bars), indicating the inflammation *in vivo* is not due to a defect in Treg-suppressive function as measured by this assay. Thus, despite the inflammation in *Brg1*^{F/F}; *CD4Cre* mice, there was no obvious defect in Treg development or function.

Treg-specific *Brg1* deletion produces early-onset, aggressive, fatal inflammation

To further explore the mechanism of autoimmunity in *Brg1*^{F/F}; *CD4Cre* mice, we deleted *Brg1* selectively in Tregs. Without the antagonizing effects of *Brg1* deletion in conventional effector T cells, the Treg-specific deletion should produce a more severe phenotype than that in *Brg1*^{F/F}; *CD4Cre* mice, if the inflammation in *Brg1*^{F/F}; *CD4Cre* mice indeed reflected a role of *Brg1* in Tregs.

To delete *Brg1* in Tregs, we crossed *Brg1*^{F/F} mice to a Cre-deleter line bearing an *Ires-Cre-YFP* cassette knocked into the 3' UTR of the *Foxp3* locus, which causes Cre-YFP fusion protein to be co-expressed with FOXP3 (Rubtsov *et al*, 2008). Since *Foxp3* is located on the X-chromosome, which undergoes random inactivation, *Brg1* was deleted in all Tregs only in *Brg1*^{F/F}; *Foxp3*^{YFP-Cre} hemizygous males and *Brg1*^{F/F}; *Foxp3*^{YFP-Cre/YFP-Cre}

Figure 1 Phenotype of *Brg1*^{F/F}; *CD4Cre* mice (KO) versus *Brg1*-sufficient littermates (WT). (A–D) Inflammatory disorder in ~5-month-old KO mice. (A) Gross appearance of colon, peripheral LN and spleen. (B) H & E staining of tissue sections of colon, liver and kidney. Arrows indicate foci of cell infiltration. (C) Cell composition of LN and spleen. Cells were stained for CD4, CD8 and TCR before TCR⁺ cells were analysed for CD4/CD8 expression. The numbers in red within the plots are total cellularity (+ / – s.d.) averaged from at least three mice. (D) Lymphocytes were either stained for CD4, CD44 and CD62L cells to quantify the abundance of effector/memory (CD44^{high} CD62L^{low}) CD4 cells (top left), or stimulated with PMA and ionomycin for 4–6 h for the analysis of cytokine induction in CD4 cells (top right). The scatter plot at the bottom summarizes the data from splenic CD4 cells. (E–G) No apparent defects in *Brg1* KO Tregs. (E) Treg frequencies in ~5-month-old mice. The data are averaged from five mice. (F) Expression of Treg markers in ~5-month-old mice. (G) *Brg1* KO did not impair the ability of Tregs to suppress CD4 cell proliferation *in vitro*. Tregs were isolated from ~4-week-old *Brg1*^{F/F}; *CD4Cre* mice. Naïve CD4 cells from WT mice were co-cultured with equal numbers of or five-fold less Tregs in the presence of mitogenic stimuli. Proliferation of CD4 cells was measured by H3-thymidine incorporation. Data are averaged from five independent experiments.

homozygous females (termed ‘KO’ thereafter), whereas the *Brg1*^{F/F}; *FoxP3*^{YFP-Cre/YFP-Cre/+} heterozygous females were mosaic, containing not only YFP⁺ but also YFP[–] Tregs and were thus healthy (see further).

We found that the KO mice invariably developed an early-onset, aggressive, fatal inflammatory disorder more severe than that in *Brg1*^{F/F}; *CD4Cre* mice and reminiscent of that seen in *Scurfy* mice bearing a germline *FoxP3* mutation that blocks Treg development (Godfrey *et al*, 1991). Specifically, starting as early as 2 weeks of age, the mice frequently began to show appreciable runting, blepharitis, alopecia, increased abdominal girth, palpable lymph nodes and pale foot pads, which became increasingly obvious and penetrant with age. Adult mice were severely runted (Figure 2A and D) and 50% of mice died within 50 days after birth, the mortality rate reaching nearly 100% by 200 days (Figure 2C), which is less severe than *scurfy* mice that died within 3–6 weeks of age in our hands. Liver necrosis was dramatic (Figure 2A), as was lymph nodes and spleen enlargement

(Figure 2A and E). Erythrocytes were visibly depleted in both bone marrow and blood, consistent with profound anaemia (Figure 2A and F). Perivascular cellular infiltration was apparent in multiple tissues (Figure 2B) with concordant accumulation of CD8 as well as B and CD4 cells in the liver (Figure 2G, right). In addition, CD8 cells accumulated in the spleen (Figure 2G, middle). The thymus were extremely small and depleted of DP cells, presumably due to distress-induced apoptosis (Figure 2H). Effector/memory CD4 cells dramatically accumulated in spleen and lymph nodes (Figure 3A and C). The CD4 cells of any single mouse overproduced one or more of the three lineage-specific cytokines (IFN-γ, IL-4 and IL-17), which suggests that *Brg1* deficiency in Tregs impaired the function of each of the three effector Treg subsets, thus pointing to a fundamental role of *Brg1* in Treg function (Figure 3B and C). Finally, CD4 cells were hyperproliferative (Figure 4E). These data confirm that *Brg1* is essential for Treg-mediated immune suppression.

Figure 2 Effects of Treg-specific *Brig1* deletion. *Brig1*^{F/F}; *FoxP3*^{YFP-Cre} males and *Brig1*^{F/F}; *FoxP3*^{YFP-Cre/YFP-Cre} females (KO) were compared with *Brig1*-sufficient, gender-matched littermates (WT) that carried one or two functional *Brig1* alleles. (A, B) Gross anatomy (A) and histology (B) of ~30-day-old males. The ears in (A) were from males different from that shown at the top of panel A. The white and red arrows in (A) indicate necrotic spots on liver and enlarged spleen, respectively. (C) Survival of males. (D) Body weight of 40-day-old males. The symbols represent individual mice. (E–H) The weight of spleen relative to that of the whole body (E), lymphocyte cellularity (F), haematocrit (G) and thymocyte composition (H) in ~30-day-old males. In H, thymocytes were stained with TCR β , CD4 and CD8 antibodies before the TCR^{-lo} and TCR^{hi} subsets were analysed for CD4/CD8 expression.

Figure 3 CD4 cells in 25- to 50-day-old KO mice. (A) Accumulation of effector memory (CD44^{high}CD62L^{low}) CD4 cells in LN and spleen. (B) Enhanced cytokine induction in CD4 cells stimulated with ionomycin (1 μ M) and PMA (1 ng/ml) for 5 h. (C) Summary of data from experiments represented in (A) and (B).

Figure 4 Tregs in 25- to 50-day-old KO mice. (A) Normal FOXP3 expression. Cells were stained for CD4 and FoxP3 before analysis. (B) KO Tregs did not express IFN- γ in hyperinflammatory environment whereas CD4 cells from the same mouse overproduced IFN- γ as expected (right). Cells were stimulated with ionomycin (1 μ M) and PMA (1 ng/ml) for 5 h before staining for intracellular FOXP3 and IFN- γ . (C) Absolute numbers of Tregs (left) and their abundance relative to CD4 cells (right) in various organs. The inset is a blown-up view of the cellularity in the liver. (D) Normal viability. Splenic lymphocytes were stained with CD4APC and annexin-PE before analysis of annexin expression in CD4⁺ YFP⁺ Tregs. (E) Proliferation. Mice were exposed to the BrdU analogue EdU for 3 days. Splenic conventional CD4 cells (CD4) and Tregs were FACS-purified based on YFP expression before analysis of EdU incorporation. (F) Expression of activation markers (including chemokine receptors) in splenic Tregs. Mice were separated into two age groups (25–29 versus 30–50 days old) based on distinct mortality rates (see Figure 2C).

***Brg1* deletion impairs Treg activation in response to self-antigens: analysis of Tregs at different stages of inflammation in mice with Treg-specific *Brg1* deletion**

We next investigated the mechanisms whereby *Brg1* promotes Treg function. We first analysed Tregs in 25–50-day-old *Brg1*^{F/F}; *FoxP3*^{YFP-Cre} males, which had developed overt inflammation. We found that *Brg1* KO Tregs expressed normal levels of FOXP3 (Figure 4A), lacked aberrant expression of proinflammatory cytokine IFN- γ , IL-4 or IL-17, were not converted to conventional CD4 cells (Figure 4B; data unpublished), and their numbers and frequencies in lymphoid organs were comparable to that in the littermate controls

(Figure 4C). Interestingly, there was a two-fold increase in total Treg numbers in the liver, suggesting some *Brg1* KO Tregs had migrated to the liver in response to inflammation (Figure 4C, left, inset) although the proportions of the Tregs relative to conventional CD4 cells remained unaltered (Figure 4C, right) due to the aforementioned accumulation of conventional CD4 cells (Figure 2G, right). Finally, *Brg1* deficiency did not affect Treg viability (Figure 4D) although Treg proliferation seemed marginally (albeit statistically insignificantly) impaired (from 5.3 ± 3.1 in WT to 2.8 ± 1.4 in KO, $P=0.07$; Figure 4E). These data do not reveal a clear defect in *Brg1* KO Treg.

As Treg activation is essential for Treg function, we examined the activation status of *Brg1* KO Tregs by analysing effector/memory-like Tregs (CD44^{high}CD62L^{low}) and multiple activation markers (CD103, CXCR3, CCR2, ICOS and CD69). Given the severe inflammation, the mutant mice should harbour more activated Tregs, and such cells should become more abundant with age. We therefore analysed both young (day 20–29) and old (day 30–50) mice; both groups had severe inflammation but the young mice were mostly viable while the old ones showed a high mortality rate (Figure 2C). As compared with WT mice, there was no increase in the frequencies of effector/memory-like or CD69⁺ Tregs even in the old KO mice, suggesting that *Brg1* deficiency indeed impaired Treg activation (Figure 4F). However, remaining markers (CD103, CCR2, CXCR3 and ICOS) were all elevated in the KO mice relative to the WT mice (Figure 4F; this upregulation of trafficking molecules is consistent with the aforementioned increase in Treg abundance in the liver shown in Figure 4C, left). The data, however, are difficult to interpret because of the severe inflammation in the KO mice; it is possible that without the inflammation, marker expression might be below the WT level, which would reveal a role of BRG1 in Treg activation. To address this, we analysed Tregs in very young (~10-day-old) *Brg1* KO mice (*Brg1*^{F/F}; *FoxP3*^{YFP-Cre} males and *Brg1*^{F/F}; *FoxP3*^{YFP-Cre/YFP-Cre} females), where outward manifestations of inflammation were unappreciable, although hepatic cellular infiltration (Figure 5A and B) and splenic accumulation of effector/memory CD4 cells (Figure 5C) had emerged. Remarkably, despite the weak inflammation, the activation markers in the *Brg1* KO Tregs were indeed expressed below the WT level (Figure 5D). Curiously, the ‘effector/memory-like’ (CD44^{high}CD62L^{low}) Tregs were only marginally decreased. However, these cells in the 10-day-old mice were paradoxically more abundant than in 25–50-day-old mice (~60–70% versus ~40%; compare Figure 5D with 4F), suggesting that in 10-day-old mice, the CD44^{high}CD62L^{low} Treg pool was heavily contaminated with non-effector/memory-like cells, which might have obscured the depletion of effector/memory-like cells in the KO mice. Finally, in agreement with defective expression of inflammatory chemokine receptors, Treg trafficking to inflamed liver seemed impaired in these mice: despite the onset of hepatitis as manifested by the cellular infiltration in the liver, hepatic Treg numbers in these mice were not increased (2300 ± 500 compared with 2900 ± 600 in WT mice; Figure 5E, top) and so the abundance of hepatic Tregs relative to total CD4 cells was decreased due to infiltration/proliferation of conventional CD4 cells (from 16.6 ± 2.2 down to 5.1 ± 0.2%; Figure 5E, bottom). Thus, in the very young KO mice, despite the mild inflammation, Tregs were hypoactivated relative to that in the WT mice, demonstrating a role of BRG1 in Treg activation.

Defective Treg activation revealed in mosaic females

To corroborate the above data, we examined the mosaic *Brg1*^{F/F}; *FoxP3*^{YFP-Cre/+} females. These mice were healthy, harbouring both YFP⁺ and YFP[−] Tregs (Figure 6A, left), with *Brg1* specifically deleted in the YFP⁺ subset (Figure 6B), thus allowing for comparison of WT and KO Tregs under identical, noninflammatory conditions.

We analysed *Brg1*^{F/F}; *FoxP3*^{YFP-Cre/+} females together with the heterozygous *Brg1*^{F/+}; *FoxP3*^{YFP-Cre/+} female littermate

controls. We found that *Brg1* deletion in *Brg1*^{F/F}; *FoxP3*^{YFP-Cre/+} females did not affect Treg viability (Figure 6C) but slightly depleted Tregs relative to the YFP[−] subset (Figure 6D, groups 3 versus 4). This depletion is consistent with impaired Treg homeostatic proliferation, as evidenced by a moderate decrease in EdU incorporation (Figure 6E). Importantly, *Brg1* deletion impaired effector/memory-like Treg production and induction of all the activation markers (Figure 6F). This activation defect may also explain the impaired homeostatic proliferation, as the latter is presumably dependent on TCR signalling and hence activation. Interestingly, in *Brg1*^{F/+}; *FoxP3*^{YFP-Cre/+} females, Treg proliferation (Figure 6E) and induction of some markers (CD69 and ICOS; Figure 6F) seemed marginally impaired in YFP⁺ Tregs as compared with the YFP[−] Treg subset, although the differences were statistically insignificant. Since the YFP⁺ cells in these mice contained only one copy of *Brg1*, the data raised the possibility that both copies of *Brg1* is required for optimal Treg activation. These data reinforce the notion that BRG1 promotes Treg activation in response to self-antigens.

Defective Treg activation during ConA-induced inflammation

We next determined whether BRG1 also promotes Treg activation in response to pathological inflammatory cues, using as a model system concanavalin A (Con A)-induced hepatitis (Tiegs, 2007). Con A triggers acute Th1-type hepatitis and induces the expression of multiple CXCR3 ligands in the liver. In response, Tregs quickly express CXCR3 (perhaps due to stimulation by Con A in combination with IFN-γ) and migrate to the liver to restrain the inflammation (Erhardt *et al*, 2011). Since *Brg1* KO Tregs must not be exposed to inflammation prior to Con A injection, we performed the experiments in *Brg1*^{F/F}; *FoxP3*^{YFP-Cre/+} females. In the experiments, the YFP[−] (WT) Tregs could theoretically serve as an internal control for YFP⁺, *Brg1*-deficient Tregs. However, as the former cells were YFP[−] *FoxP3*⁺, a combination of FOXP3 staining and YFP fluorescence was needed for their identification, which proved technically difficult especially for the cells isolated from the liver (not shown). One option would be to use CD25 to identify the YFP[−] Tregs. However, CD25 is also expressed in conventional CD4 cells where its expression is presumably subject to influences by Con A, thus confounding data interpretation. We therefore used the *Brg1*^{F/+}; *FoxP3*^{YFP-Cre/+} female littermates as controls, as these mice contained *Brg1*-sufficient Tregs marked by YFP, which bypassed the need for FOXP3 or CD25 staining.

Our analysis was focused on CXCR3⁺ Tregs, the central player in this hepatitis model (Erhardt *et al*, 2011). As expected from Figure 6F, prior to Con A injection, the frequencies of *Brg1* KO YFP⁺ CXCR3⁺ Tregs were ~2-fold lower in *Brg1*^{F/F}; *FoxP3*^{YFP-Cre/+} mosaic females than the WT counterpart in the littermate controls (Figure 7A, left and Figure 7B, top). We then injected Con A and analysed CXCR3 induction 48 h thereafter, at a time when Treg numbers in the liver had peaked (Supplementary Figure S2). Before Con A injection, CXCR3 was expressed in ~21% of YFP⁺ WT Tregs in the spleen, and the value was increased to ~42% following Con A injection (Figure 7A; Figure 7B, top). In other words, the YFP⁺ WT Tregs that lacked CXCR3 expression comprised 79% of total Tregs before Con A injection, but only

Figure 5 Treg hypoactivation in ~10-day-old KO mice. (A) Cell infiltration (arrow) in liver in *Brg1* KO mice. (B) Numbers of lymphocytes recovered from the liver. The inset is a blown-up view of the CD4 and CD8 cellularity. (C) Accumulation of effector/memory CD4 cells in the spleen. A representative experiment was shown at the top, and the data summarized at the bottom. (D) Defective Treg activation. Shown are the frequencies of splenic Tregs expressing various surface markers. (E) Relative depletion of Tregs in the liver.

Figure 6 Tregs in mosaic *Brg1*^{F/F}; *FoxP3*^{YFP-Cre/+} (*F/F*) adult females and the littermate control *Brg1*^{F/+}; *FoxP3*^{YFP-Cre/+} (*F/+*) females. (A) YFP versus CD25 expression in total CD4 cells. (B) qPCR measuring *Brg1*^F abundance in Tregs. (C) Viability of YFP⁺ Tregs. Splenic Tregs were stained for CD4 and annexin before analysis of annexin expression in CD4⁺ YFP⁺ Tregs. (D–F) Abundance (D), proliferation (E) and activation (F) of YFP[−] versus YFP⁺ Tregs.

58% after the injection. This indicates that 21% (79–58%) of YFP[−] Tregs had upregulated CXCR3 following Con A injection. Since the CXCR3[−] Treg subset comprised 79% of YFP⁺

Tregs before Con A injection, Con A must have induced CXCR3 on 27% (21/79) of CXCR3[−] YFP⁺ WT Tregs. In contrast, for the KO counterpart, CXCR3 was expressed

Figure 7 *Brg1* KO impaired CXCR3 induction and Treg trafficking following Con A stimulation. (A) CXCR3 expression in YFP⁺ Tregs in the spleen (top) and liver (bottom) in the absence (left) or 2 days after (right) ConA injection. WT and KO Tregs were YFP⁺ cells in *Brg1*^{F/F}; *FoxP3*^{YFP-Cre/+} and *Brg1*^{F/F}; *FoxP3*^{YFP-Cre/+} females, respectively. (B) The data in (A) were summarized from multiple experiments to show the percentages of CXCR3⁺ subset among total YFP⁺ Tregs (top) and the mean fluorescence intensity (MFI) of CXCR3 expression in the CXCR3⁺ subset (bottom). (C) Same as B, except that the absolute numbers of Tregs are displayed.

on ~8 and ~17% YFP⁺ KO Tregs before and after Con A injection, respectively, indicating Con A had induced CXCR3 in only 10% of CXCR3⁺ YFP⁺ KO Tregs as compared with 27% of WT counterparts (Figure 7A and Figure 7B, top). Thus, *Brg1* deletion led to a 2.7-fold reduction in the CXCR3 induction frequency in CXCR3⁺ YFP⁺ Tregs. Furthermore, surface CXCR3 abundance (as measured by MFI) on the *Brg1* KO Tregs that had managed to express CXCR3 was lower (~1.7-fold on average) at the single cell level both before and after Con A injection (Figure 7A and Figure 7B, bottom). Thus, *Brg1* deficiency compromised both the probability and magnitude of CXCR3 induction in the resting state and following Con A stimulation. As a result, at 48 h following Con A injection, there were ~109 000 CXCR3⁺YFP⁺ WT Tregs in the spleen, but only ~28 000 KO counterparts (Figure 7C), indicating a four-fold reduction in the absolute numbers of KO Tregs after Con A injection in the spleen. CXCR3 is crucial for Treg trafficking to the liver during Con A-induced hepatitis. The fact that fewer CXCR3⁺YFP⁺ KO Tregs were present in the spleen predicts that fewer CXCR3⁺YFP⁺ KO Tregs can be recruited to the liver during the hepatitis. Indeed, before Con A injection, ~600 and ~200 CXCR3⁺ WT and KO YFP⁺ Tregs were present in the liver, respectively (Figure 7C, top). Two days following Con A injection, the numbers of hepatic WT CXCR3⁺ YFP⁺ Tregs had increased from 600±380 to 7350±3080, suggesting ~6750 WT YFP⁺ CXCR3⁺ Tregs had been recruited during this time (Figure 7C, top), and these new recruits largely account for the increase in the numbers of total YFP⁺ cells in the liver (from 2000 to 11 300, Figure 7C, bottom), as expected. In contrast, following Con A injection, KO YFP⁺ CXCR3⁺ Tregs were increased by only ~730 (from 230±130 to 960±540; Figure 7C, top). Thus, nine-fold (6750 versus 730) fewer YFP⁺ CXCR3⁺ KO Tregs were recruited to the liver, confirming our prediction. Interestingly, this nine-fold reduction in the number of recruited KO Tregs was disproportional to the aforementioned four-fold reduction in the num-

bers of YFP⁺ CXCR3⁺ KO Tregs in the spleen. Since the KO CXCR3⁺ Tregs showed impaired surface CXCR3 expression (in the liver as in the spleen; Figure 7B, bottom), the lowered CXCR3 expression presumably impeded trafficking of CXCR3⁺ Tregs. The impediment in the trafficking of the CXCR3⁺ Tregs may explain the disproportional reduction in the hepatic versus splenic KO CXCR3⁺ Tregs. Perhaps for the same reason, at the basal state before Con A injection, hepatic KO CXCR3⁺ Tregs (200 cells versus 600 WT counterparts) were also disproportionately depleted as compared to the splenic Tregs (12 600 KO versus 29 400 WT cells). These data demonstrate that *Brg1* deletion impaired Treg activation in response to pathological inflammatory cues, just as it impaired Treg activation by self-antigens.

In the discussion above, we have assessed Con A-induced Treg recruitment based on the absolute numbers of recruited Tregs rather than fold increases in hepatic Treg abundance relative to the basal levels, as the fold change is confounded by the basal number of CXCR3⁺ Tregs and thus cannot directly and accurately represent the effect of Con A. It is also noteworthy that the activation and trafficking defects in *Brg1* KO Tregs were not caused by the presence of the WT Tregs in the mosaic mice; the WT Tregs could compete with the KO Tregs thus helping reveal their intrinsic defects, but could never create such defects.

Brg1* regulates TCR target genes *in vitro

The experiments described above were carried out *in vivo*. To directly test the role of BRG1 in Treg activation, YFP⁺ KO Tregs lacking prior inflammatory exposure were isolated from *Brg1*^{F/F}; *FoxP3*^{YFP-Cre/+} mosaic females and stimulated with anti-CD3/CD28-coated beads in the presence of IL-2. YFP⁺ Tregs from *Brg1*-sufficient mosaic females were used as a control. To ensure the Tregs were not contaminated with conventional CD4 cells, Tregs were purified to near homogeneity using anti-CD25 magnetic beads followed by

Figure 8 BRG1 regulated TCR target genes *in vitro*. (A) YFP⁺ Tregs isolated from *Brg1*^{F/F}; *FoxP3*^{YFP-Cre/+} (WT) or *Brg1*^{F/F}; *FoxP3*^{YFP-Cre/+} (KO) females were untreated (–) or stimulated (+) with anti-CD3/CD28 for 2 h (for *Nur77*) or 48 h (for the remaining genes). mRNA was quantified by RT-PCR, normalized to β -actin mRNA and plotted relative to that in activated WT Tregs, the latter set as 100 (dotted line). Data were pooled from 2–3 independent trials. *Gad*, *Gadd45* γ . (B) BRG1 binds the *Cxcr3* and *Ccr2* loci. Freshly isolated YFP⁺ *Brg1*-sufficient Tregs were subjected to ChIP assays using J1 anti-BRG1 antibody or rabbit IgG control, and the abundance of the precipitated DNA sequences was plotted relative to that in the input; IgG-precipitated DNA was often below the detection limit. *RNA Pol N* (De *et al*, 2011) and *Gapdh* served as a positive and negative control, respectively. The locus structure is depicted at the left, where the blue arrows indicate the positions of the PCR amplicons in the ChIP assays. A replica experiment is shown in Supplementary Figure S3B.

electronic sorting for YFP⁺ cells. Ideally, the experiment should use Tregs lacking activation marker expression, but this is not feasible because even ‘naïve’ (CD103[–] CD44^{low} CD62L^{high}) Tregs can express activation markers (Huehn *et al*, 2004).

Tregs were stimulated for 2, 12 or 48 h before analysis of gene expression. We examined all the activation marker genes that we have characterized *in vivo* as well as multiple known or suspected TCR target genes in Tregs (McHugh *et al*, 2002; Park *et al*, 2010; Moran *et al*, 2011), but only 10 genes showed reproducible changes in our hands (Figure 8A). The expression levels of these genes before TCR stimulation were comparable between WT and KO Tregs, and TCR stimulation induced all these genes in WT Tregs, but with different kinetics (Figure 8A). Thus, *Nur77* induction was rapid and transient, reaching the plateau within 2 h after stimulation but had returned to baseline by 48 h, whereas the induction of the remaining genes was not detectable at 2 h, variable at 12 h and became stable at 48 h. Importantly, *Brg1* KO impaired the induction of all chemokine receptor genes (*Cxcr3*, *Cxcr5*, *Ccr2*, *Ccr6* and *Ccr8*), *Gadd45* γ and *Socs2* (Figure 8A). The effects mirror the activation defects *in vivo*, arguing for a role of BRG1 in facilitating Treg activation in response to TCR signalling. However, the *in vitro* data must be interpreted with caution as it was unable to fully recapitulate the *in vivo* phenomena: while *Brg1* KO impaired *Icos* induction *in vivo*, BRG1 was dispensable for *Icos* induction *in vitro* (Figure 8A). Furthermore, increasing the dose of anti-CD3/CD28 or addition of IFN- γ failed to rescue the activation defects (preliminary data).

As mentioned above, the expression levels of TCR target genes were comparable between freshly isolated WT and *Brg1* KO Tregs, which apparently contradicts the fact that the former Treg population harboured ~20% of Tregs expressing markers such as *Cxcr3*, *Ccr2* and *Icos* while the latter only ~10% (Figure 6F). However, RT-PCR quantified the RNA in the entire Treg population, including both the marker⁺ and the marker[–] subsets. The latter subset, which constituted the majority of Treg population, might contain basal levels of RNA detectable by RT-PCR. Furthermore, the former, marker⁺ subset only weakly expressed the markers even in WT Tregs. Thus, the overall difference in mRNA abundance between freshly isolated WT and *Brg1* KO populations might be <2-fold, which would be difficult to detect by RT-PCR.

A potential caveat in the experiment described above is that YFP⁺ WT Tregs in the *Brg1*-sufficient mosaic females harboured two-fold more effector/memory-like cells than the YFP⁺ *Brg1* KO Tregs in *Brg1*^{F/F}; *FoxP3*^{YFP-Cre/+} mosaic females (Figure 6F). Since effector/memory-like cells presumably react more robustly to TCR stimulation, the apparent defect in TCR signalling in *Brg1* KO Tregs may simply reflect the depletion in effector/memory-like Tregs. However, the two-fold reduction in the effector/memory-like cells could not account for the more severe defects in the induction of some of the BRG1-dependent genes (e.g., *Cxcr3*, *Cxcr5*, *Ccr2*, *Ccr6* and *Gadd45* γ). Furthermore, the induction of these five genes was similarly defective in YFP⁺ Tregs isolated from ~21-day-old *Brg1*^{F/F}; *FoxP3*^{YFP-Cre} males (Supplementary Figure S3A), where overt inflammation had developed and the abundance of effector/memory-like Tregs was compar-

able to WT mice (Figure 4F). The data thus confirm the roles of BRG1 in TCR signalling at least for these five genes. We conclude that BRG1 regulates a subset of TCR target genes *in vitro*.

Brg1 binds the Cxcr3 and Ccr2 loci in Tregs

We sought to determine whether BRG1 directly regulates the target genes. We focused on *Cxcr3* and *Ccr2* because their expression was strongly BRG1-dependent both *in vitro* and *in vivo*. Several BRG1-binding sites have been mapped at each gene in conventional CD4 cells, including regions ~3- and ~30-kb upstream of the *Cxcr3* and *Ccr2* transcription start sites, respectively (Figure 8B, left) (De *et al*, 2011). We consistently detected BRG1 at both sites in YFP⁺ Tregs (Figure 8B, right; Supplementary Figure S3B), which suggests that BRG1 directly regulates *Cxcr3* and *Ccr2* in Tregs.

BRG1 ATPase activity is partially dispensable for BRG1 function

Using a genetic method that can produce littermates bearing either *Brg1* KO or *Brg1* ATPase-dead K/R point mutation (PM), the former completely eliminating BRG1 function while the latter selectively abolishes ATP-dependent remodelling, we previously found that BRG1 harbours non-classical, remodelling-independent function (RIF) during early T-cell development (Jani *et al*, 2008). To explore the role of RIF in Tregs, we compared the phenotypes of Treg-specific KO versus PM mice. We found dramatic differences in the mortality rates: while the KO mice mostly died by 120 days, ~75% PM littermates survived at least 200 days (Figure 9A). Furthermore, although PM mice (14.2 ± 3.5 g on day 40) were very runted as compared with the WT controls (21.8 ± 2.0; $P < 0.001$), they were heavier than the KO littermates (11.6 ± 2.8; $P = 0.02$) (Figure 9B). In addition, in PM mice, lymphadenopathy, splenomegaly and liver inflammation were mild and kidney inflammation undetectable (Figure 9C and D). Remarkably, despite the absence of severe inflammation in the PM mice, Tregs in the PM were as hyperactivated as the KO littermates (Figure 9E, top), suggesting that Tregs in the PM mice remained highly sensitive to inflammatory signals. Indeed, even in mosaic mice, under noninflammatory condition, Tregs bearing the *Brg1* PM showed normal expression of CD103, CXCR3 or CCR2 (Figure 9E, bottom), and Treg abundance was not significantly reduced (Figure 9F). However, in the mosaic mice, *Brg1* PM did deplete effector/memory-like Tregs and impair CD69 and ICOS expression to the same extent as *Brg1* KO (Figure 9E, bottom), indicating that classic ATP-dependent chromatin remodelling is important for these aspects of Treg activation (only) under strictly physiological condition. Of note, the discrepancy in the phenotypes caused by *Brg1* PM versus KO is not an artifact resulting from inefficient *Brg1*^F deletion in the PM mice (Figure 9G). Finally, while the mice deleting *Brg1* in all $\alpha\beta$ T cells (using the CD4Cre deleter) developed late-onset inflammation as described before (Figure 1), the littermates bearing the BRG1 PM in these T cells showed little phenotype (Supplementary Figure S4), consistent with the phenotype of mice bearing Treg-specific PM. Collectively, these data demonstrate that BRG1 ATPase activity is partially dispensable for BRG1 to suppress autoimmunity, thus exposing the role of RIF in immune tolerance.

Discussion

This study demonstrates that BRG1 promotes Treg activation and immune tolerance, which overweighs its proinflammatory role in conventional T cells. *Brg1* KO Tregs can be partially activated by persistent, severe inflammation, but this activation is not sufficient to stop the inflammation. Finally, the classic ATP-dependent chromatin-remodelling activity is partially dispensable for BRG1 to promote Treg activation and immune tolerance.

Facilitation of Treg activation by BRG1: biological importance, potential mechanisms and cell-type specificity

KO Tregs were invariably hypoactivated relative to WT Tregs under comparable conditions, whether in the 10-day-old mice, in the normal or inflamed mosaic mice, or *in vitro* following TCR stimulation. These data reveal an activation defect in the KO Tregs. While the full spectrum of the defect *in vivo* remains to be determined, which is technically challenging because the defects should be analysed at the single cell level as mentioned in the Results section, it is already clear that *Brg1* KO impaired the induction of multiple types of functionally important genes, including chemokine receptor genes, *Cd103* and *Icos*, which are involved in Treg trafficking, retention and the suppressive potential, respectively (Suffia *et al*, 2005; Campbell and Koch, 2011; Kornete *et al*, 2012). In addition, *Brg1* KO impaired Treg proliferation *in vivo*, which may also be secondary to a defect in Treg activation. Finally, *in vitro* assays suggest that *Brg1* regulated several other TCR target genes, including *Gadd45 γ* and the FoxP3-dependent gene *Socs2* (Sugimoto *et al*, 2006). Thus, *Brg1* KO compromised multiple activation-dependent events in Tregs. These various defects may conspire to cause or at least contribute to the onset of the inflammation. Remarkably, as the inflammation in the KO mice persisted and progressed, *Brg1*-deficient Tregs became increasingly activated. However, CXCR3 and CCR2 expression in 20–29-day-old mice was only marginally above the WT level despite the severe inflammation in these mice, and furthermore, the abundance of effector/memory-like and CD69⁺ Tregs remained at the WT level even in 30–50-day-old mice. Thus, although strong and persistent inflammatory cues could activate KO Tregs to some degrees, the activation level was disproportional to the severity of the inflammation. Indeed, this level was not higher than in the Tregs in the PM mice lacking severe inflammation, again arguing that the activation level in the KO mice failed to ‘match’ the severity of inflammation. While this activation level might suffice to control mild inflammation, it is presumably insufficient to ‘catch up with’ and stop the ongoing severe inflammation in these mice. Furthermore, even if Tregs could be fully activated by the severe inflammation, it may be too late to benefit the mice, as inflammation-induced tissue damages might have become irreversible in 20–50-day-old mice. We therefore think that BRG1 promotes immune tolerance at least in part by facilitating Treg activation in response to environmental cues, thus allowing Tregs to promptly and robustly react to incipient inflammation and nip it in the bud; while the KO Tregs eventually became somewhat activated following persistent and strong provocation, this response would be too late and too weak to control the already severe inflam-

Figure 9 Phenotype of mice bearing Treg-specific *Brg1* PM. (A–D) Survival (A), body weight at 40 days of age (B) and various organs (C, D) at ~30 days of age. All mice were males. A and B are identical to Figure 2C and Figure 2D, respectively, except for the inclusion of *Brg1*^{F/O}; *FoxP3*^{YFP-Cre} point mutants (PM). Arrow in D indicates marginal infiltration in the liver. (E) Treg activation in 20- to 50-day-old mice (top) and in adult mosaic mice (bottom), which are identical to Figure 4F and Figure 6F, respectively, except for the inclusion of the PM mice. Note that in the plots at the top, the mice are not separated into different age groups as in Figure 4F due to limiting sample sizes of the PM mice. F/O, mosaic PM females (*Brg1*^{F/O}; *FoxP3*^{YFP-Cre/+}). (F) Treg abundance in adult mosaic females. The plot is identical to Figure 6D except for the inclusion of PM mice (*Brg1*^{F/O}; *FoxP3*^{YFP-Cre/+}). (G) PCR analysis of *Brg1*^F (F) and *Brg1*^O (O) in tail (lanes 1–2) and YFP⁺ Tregs (lanes 3–4) from *Brg1*^{F/O}; *FoxP3*^{YFP-Cre/+} mosaic females. The two alleles were detected by a primer pair flanking the 3′-LoxP site in *Brg1*^F and that amplifying a fragment specifically present in *Brg1*^O, respectively. The asterisk indicates co-amplified internal controls. The data indicate efficient *Brg1*^F deletion concomitant with *Brg1*^O conversion. The assays were performed essentially as described (Jani et al, 2008).

mation. Obviously, the activation defects do not exclude additional defects that might contribute to the autoimmune disease in the KO mice, and future studies are needed to identify such potential additional defects. Of note, while the BAF complex associates with FOXP3 (Li et al, 2006, 2007; Rudra et al, 2012), the significance of this interaction is unclear, as *Brg1* deletion did not affect FOXP3-dependent processes such as Treg development, expression of Treg signature genes and prevention of Tregs conversion into effector T cells.

Our *in vitro* assays suggest that BRG1 regulates a subset of TCR target genes in Tregs. Multiple mechanisms that are not mutually exclusive might be involved in this regulation. In conventional CD4 cells, BRG1 is rapidly (within minutes) recruited to nuclear matrix upon TCR signalling, suggesting that BRG1 might exert an acute, global, nonspecific effect on the nuclear architecture that might influence the induction of all TCR target genes (Zhao et al, 1998). In addition, BRG1 binds both the *Cxcr3* and *Ccr2* loci in Tregs, suggesting that BRG1 can directly and specifically regulate some of the target genes. Finally, BRG1 might act indirectly, by fine-tuning the

expression of intermediate molecules in the TCR signalling pathways, and this fine-tuning may be required for the optimal induction of some BRG1 target genes. Thus, BRG1 may use distinct mechanisms to regulate different target genes.

The roles of BRG1 in Treg activation may be divergent from that in conventional CD4 cells, given the divergence in the activation mechanisms between the two cell types (see Introduction). Indeed, although *Brg1* RNAi impairs Th1/Th2 differentiation *in vitro*, it does not seem to impair CD4 cell proliferation, a hallmark of activation (Zhang and Boothby, 2006; Wurster and Pazin, 2008). Furthermore, while BRG1 promotes Th1/Th2 differentiation in CD4 cells by remodelling *Il4* and *Ifng* chromatin structure, respectively, remodelling activities were partially dispensable for BRG1 to promote Treg function, reinforcing the notion about cell-type specificity in BRG1 action.

Remodelling Independent Function (RIF) of BRG1

BRG1 is generally assumed to act by ATP-dependent remodelling of nucleosome structure. However, we previously found that eliminating this remodelling activity fails to com-

pletely abolish BRG1 function during early T-cell development: while the remodelling activity is essential for BRG1 to promote survival and development of early thymocytes, it is dispensable for BRG1 to regulate CD4 expression during DN to DP transition (Jani *et al*, 2008). We now demonstrate that as a whole, the remodelling activity was partially dispensable for BRG1 to promote Treg function and suppress autoimmunity, because overall, the BRG1 ATPase PM mice displayed an intermediate phenotype between the WT and KO mice. Interestingly, different components of the phenotype were differentially affected by the point mutation, although for the most part, the defects in the PM mice were rather weak. Specifically, while severe runting was present in both KO and PM mice, lifespan reduction (within the time window analysed), liver inflammation, lymphadenopathy and splenomegaly were dramatic in KO but mild in the PM mice, and furthermore, kidney inflammation was totally absent in the PM mice. Thus, chromatin remodelling by BRG1 is essential for BRG1 to regulate body weight, partially/largely dispensable for controlling lifespan, liver inflammation, lymphadenopathy and splenomegaly, and fully dispensable for repressing kidney inflammation. At the transcription level, the PM impaired the induction of some (*Icos* and *Cd69*) but not other (*Cd103*, *Cxcr3* and *Ccr2*) activation markers in mosaic mice, indicating that RIF is necessary and sufficient for BRG1 to regulate a subset of target genes. This conclusion has been reached before when we showed, as mentioned above, that RIF alone can regulate CD4 expression during DN→DP transition (Jani *et al*, 2008), but our data in the current paper reveal a significant role of RIF in immune tolerance, thus highlighting for the first time the biological importance of RIF. Of note, the role of RIF may have been underestimated in the current study, because the defects in the PM mice were compared to the *Brg1*-sufficient control mice some of which carried two copies of the functional *Brg1* allele in Tregs (see Figure 2 legend), whereas in the PM mice, the ATPase-dead BRG1 mutant was expressed from the single-copy mutant allele (Jani *et al*, 2008). Thus, the BRG1 abundance in the control mice was perhaps higher than that of the ATPase-dead mutant in the PM mice, which might have exaggerated the defects in the PM mice, leading to the overestimation of the importance of the BRG1 ATPase activity. The molecular basis of RIF is unclear, but may reflect a role of BRG1 in organizing nuclear architecture, as suggested by rapid targeting of BRG1 to nuclear matrix following TCR stimulation (Zhao *et al*, 1998). Such global organization might entail the functions of the BAF complex in facilitating actin polymerization (Rando *et al*, 2002) and large-scale chromatin movement (Hu *et al*, 2008), which are conceivably independent of the traditional nucleosome remodelling activity of BRG1.

Interesting, the H3K27 demethylase *Jmjd3* is recently shown to promote *Ifng* expression and Th1 differentiation independently of its catalytic activity, because point mutations that abolish *Jmjd3* catalytic activity do not affect its function (Miller *et al*, 2010). Similarly, P53 point mutants unable to induce cell cycle arrest, apoptosis or senescence unexpectedly retain substantial tumour-suppressing activities (Li *et al*, 2012b). In general, proteins are multi-functional, and our genetic strategy of producing littermates bearing either KO or PM is useful for dissecting these functions.

Materials and methods

Mice

Floxed *Brg1* (*Brg1^{F/F}*) mice (Chi *et al*, 2003) and *Brg1⁰* mice bearing conditional *Brg1* PM (Jani *et al*, 2008) were mated with *CD4Cre* (Lee *et al*, 2001) or *FoxP3^{YFP-Cre}* (Rubtsov *et al*, 2008) mice to mutate *Brg1* in all $\alpha\beta$ T-cell lineages or Tregs only, respectively. The mice were maintained on the C57BL/6 background.

Histology

Tissues were fixed in the Prefer fixative. Paraffin embedding and H&E staining were performed by the histology service at Yale Research Histology.

Cytokine production in T cells in vitro

Lymphocytes were isolated from spleen and lymph nodes in complete RPMI. About 0.2 million cells were incubated for 5 h at 37°C with PMA (1 ng/ml), ionomycin (1 μ M) and Golgistop (3 mM) before the analysis. Cells incubated with Golgistop alone were used as a negative control. Intracellular staining of cytokines was performed according to the protocol from Ebioscience.

EdU incorporation

For the experiments in Figure 4E, EdU (300 μ g, Invitrogen) was injected i.p. once a day for three consecutive days to ~30-day-old KO (*Brg1^{F/F}*; *FoxP3^{YFP-Cre}*) mice or littermate controls. On day four, lymphocytes were harvested from the spleen, CD4 cells magnetically enriched, stained with anti-CD4-PerCP before purification of conventional CD4 cells and Tregs via electronic sorting based on YFP expression. EdU incorporation was then determined using the Click-it EdU staining (Invitrogen). The separation of CD4 cells and Tregs before EdU staining was necessary because the Click-it chemistry quenches the YFP fluorescence. The EdU incorporation in Tregs from the mosaic mice (Figure 5E) was similarly determined, except that the CD4 cells were stained with anti-CD4-PerCP together with anti-CD25-FITC before sorting and subsequent EdU staining of YFP[−]CD25⁺ and YFP⁺CD25⁺Tregs.

Treg-suppressive function in vitro

PLNs and spleens were harvested from ~6-week-old mice and cells pooled. Naïve CD4 cells (from WT mice) and Tregs (from WT and *Brg1^{F/F}*; *CD4Cre* mice) were sorted using CD4⁺CD25[−]CD45RB^{hi} and CD4⁺CD25⁺CD45RB^{low} as markers, respectively. The purity of sorted cells exceeded 95%, and 95–98% of the sorted 'Tregs' expressed Foxp3 (not shown). About 2×10^4 naïve CD4 cells were mixed with varying numbers of Tregs (CD4⁺CD25⁺) in the presence of 8×10^4 irradiated antigen-presenting cells, the latter prepared by removing CD4 and CD8 cells from splenocytes and lymph node cells followed by lethal X-ray irradiation. Cells were stimulated with soluble α -CD3 (1 μ g/ml) for 48 h. ³H-thymidine (1 μ Ci) was then added, and ³H incorporation measured 24 h thereafter using a β -counter.

Hepatitis induction and lymphocyte analysis

Six-week-old *Brg1^{F/F}*; *FoxP3^{YFP-Cre}*/+ or *Brg1^{F/+}*; *FoxP3^{YFP-Cre}*/+ females were injected via the tail vein with 1 mg/ml Concanavalin A Type IV (Sigma 11028-71-0, diluted in PBS) at 10 μ g/g body weight. Mice were sacrificed 48 h later and lymphocytes collected from lymph nodes, spleen and liver. Liver lymphocytes were isolated as described (Zenewicz *et al*, 2007). Briefly, liver tissues were mechanically disrupted in 5 ml RPMI and digested for 40 min with 0.01 mg Collagenase IV (Sigma C-5138) and 0.2 μ g DNase I (Sigma D4527). The samples were washed with 45 ml RPMI and debris removed by centrifugation at 300 r.p.m. for 3 min. The cells were pelleted by centrifugation at 1600 r.p.m. for 10 min, and resuspended in 1 ml RPMI media plus 4 ml gradient buffer, the latter comprising Optiprep, Tricinc buffer (30 mM Tricinc-NaOH pH 7.4, 0.85% NaCl) and PBS at 2:1:1.4 ratio (volume). Cells in the gradient buffer were transferred to 15 ml conical tubes, overlaid with 1 ml RPMI media and centrifuged for 20 min at 1650 r.p.m. at 4°C without acceleration or brake. Cells at the interface were collected and washed with 15 ml RPMI media. Contaminating RBC were then lysed before analysis of the lymphocytes.

Treg activation in vitro

CD25⁺CD4 cells from adult *Brg1^{F/F}*; *FoxP3^{YFP-Cre}*/+ or *Brg1^{F/+}*; *FoxP3^{YFP-Cre}*/+ females were first magnetically isolated with a Treg

purification kit (Invitrogen, cat no 113.63D) prior to FACS sorting for YFP⁺ subset, the purity of sorted cells exceeding 99%; the sorting step was included to ensure that Tregs were not contaminated with CD25⁺ conventional CD4 cells. About 0.1–0.2 million sorted cells were incubated with equal numbers of anti-CD3/CD28 dynal beads (Invitrogen) in the presence of 2 U/ μ l recombinant IL-2 (Peprotech). Cells were harvested at indicated times and RNA purified with the RNeasy kit (Qiagen). Target genes were quantified by real-time, one-step RT-PCR using the QuantiTect SYBR Green RT-PCR kit (Qiagen). The primer sequences are available upon request.

BRG1 ChIP

YFP⁺ Tregs were isolated from *Brg1*-sufficient adults as described above. BRG1 ChIP was performed essentially as described (Jani *et al*, 2008). Briefly, cells were washed once in PBS and then fixed with 6 μ g/ml dimethyl adipimidate for 90 min at room temperature. Cells were washed again with PBS and then fixed with 1% paraformaldehyde at room temperature for 5 min. Cells were resuspended in RIPA (0.8 M NaCl) at a final concentration of 40 million cells/ml. Fixed cells were sonicated for 45–60 min (30' on/30' off, at the high intensity) in a Bioruptor sonicator to shear chromatin to ~200 basepair fragments. One to two million cells were diluted with equal volume of RIPA (0 M NaCl), and chromatin precipitated. ChIP DNA and input DNA were quantified by real-time PCR. Primers for amplifying *Cxcr3*- and *Ccr2*-binding sites are: (*Cxcr3*) 5'-TTGGCTAGGTAGTGCCTCTGACCTT-3' and 5'-TGCCAGTTAGTGGCTTGCTC-3'; (*Ccr2*) 5'-AGGAGTGGGA GGTACAGGAAGT-3' and 5'-TGCCACAGAAGGGAGGTGCTCAT-3'.

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Sample size and statistical analysis

Unless otherwise noted in the figure legends, for any experiment, at least three independent experiments were performed, and at least three mice total per group were analysed. *P*-values were calculated by *t*-test.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contribution: BHC and HH analyzed mice with Treg-specific Brg1 mutation, while AJ and YW analyzed mice with T-cell-specific Brg1 mutations. RF and TC directed the study. TC conceived the project and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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