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Alternative NF-κB signaling controls peripheral homeostasis and function of regulatory T cells

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ABSTARCT

Regulatory T cells (Tregs) maintain immune homeostasis and play an important role in tissue regeneration after injury. Mutations affecting development or homeostasis of Tregs lead to immune pathologies in humans and are often fatal in mouse models. Although the pathways required for Treg development are being increasingly characterized, factors crucial for Treg homeostasis are not completely understood. Previously we have found a role for alternative NF-κB pathway in restricting T cell activation and Th17 differentiation. Here, by using the mouse model of uncontrolled alternative NF-κB signaling we identify a crucial intrinsic role of RelB signaling in regulating homeostasis and competitive fitness of Tregs. The failure of $p100^{-/-}$ Tregs to maintain the population of effector Tregs and efficiently suppress immune reactions results in lethal multiorgan Th1-mediated inflammation in $Rag1^{-/-}$ recipients. This inflammation is combined with severe lymphopenia and could be rescued by adoptive transfer of wild type Tregs. Thus in addition to its role in Th17 differentiation, RelB acts as a potent inhibitor of Treg effector functions. Our results point to RelB as a potential therapeutic target for Treg manipulation.

1. Introduction

Regulatory CD4 T cells represent the key immune cell population, controlling unwanted inflammation towards self-antigens or commensals. The unique feature of regulatory T cells is their expression of Foxp3 transcription factor, which is essential for their identity and sufficient to provide their suppressive function. Foxp3 expressing Tregs are crucial for maintaining lifelong protection from fatal autoimmunity by suppressing immune cells, including CD4, CD8, B cells and antigen presenting cells (Schmidt et al., 2012). Recent progress in T cell biology have significantly improved our understanding about this cell type. Now Tregs are seen not only as mere suppressors, but rather as sentinel cells, which maintain homeostasis of the non-lymphoid tissue (Arpaia et al., 2015; Panduro et al., 2016). Currently molecular mechanisms of Treg development are well-characterized (Kitagawa et al., 2015; Kitagawa and Sakaguchi, 2017). Specifically the role of NF-κB signaling in Treg biology is well-defined (Fulford et al., 2015; Luu et al., 2017; Vasanthakumar et al., 2017; Fischer et al., 2017. However most of our knowledge on NF-κB in Tregs comes from the studies of classical NF-κB signaling mediated through RelA or c-rel (Messina et al., 2016; Grinberg-Bleyer et al., 2017; Schuster et al., 2017 but the role of their counterpart — RelB in Tregs is not known. Traditionally NF-κB signaling is separated into classical and alternative pathways. The classical NF-κB acts through the degradation of IκBα and nuclear translocation of RelA-p50 or c-rel-p50 complexes. The alternative pathway requires inducible degradation of p100 (NF-κB2) to p52 and subsequent p52-RelB translocation to the nucleus (Sun, 2011). To date, a number of TNF-receptor family members have been described to signal *via* alternative NF-κB, including LTβR, CD40, OX40, CD30 and others (Sun, 2011). To address the role of RelB in Tregs we used a mouse model that lacks the C-terminal end of the p100 molecule but still possesses a functional p52 ($p100^{-/-}$ mice) which leads to the unrestricted alternative NF-κB signaling *via* RelB (Ishikawa et al., 1997; Krljanac et al., 2014a). This model allows to address the outcome of the distal receptor-independent activation of RelB. Previously we have shown that such mice have defects in T-cell-independent response and a block in Th17 development (Krljanac et al., 2014a; Koliesnik et al., 2017). Here we report for that overt alternative NF-κB negatively impacts regulatory

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T cell peripheral homeostasis and phenotype as well as their suppressive function. This functional defect is compensated by host lymphocytes in an immunocompetent host, but is unleashed into a fatal multiorgan inflammation in a Rag1$^{-/-}$ host. Our study provides additional cell-intrinsic role for RelB in T cell biology making it a potential therapeutic target in immunotherapy.

2. Materials and methods

2.1. Mice

p100$^{-/-}$ (Ishikawa et al., 1997) and wild type (wt) mice were maintained on a C57BL/6 background. All mice were housed and bred under specific pathogen free conditions and fed a standard rodent chow. All animal experiments complied with ethical standards of the German Animal Welfare Act. Bone marrow transfer experiments were performed under license Reg.-Nr. 03-007/12.

2.2. Bone marrow transplantation

Bone marrow chimera (BMC) generation was described previously (Krljanac et al., 2014a). Briefly, bone marrow was isolated from hind limbs of p100$^{-/-}$ mice and wild-type littermates. Red blood cells were lysed and 4–6 × 10^6 bone marrow cells in PBS were intravenously injected into lethally gamma-irradiated (12 Gy split dose) 1.5–3 months old wt C57BL/6 or Ly5.1 recipients. Irradiation for Rag1$^{-/-}$ mice was a single dose of 9 Gy. Experiments with BMC were performed 2–4 months after reconstitution.

2.3. Flow cytometry

Single cell suspensions from spleen, lymph nodes and thymus were prepared by mechanical disruption in FACS buffer (PBS, 0.5% bovine serum albumin, 2 mM EDTA). Cells were acquired on FACS Canto II cytometer. Recorded data were analyzed with FlowJo 7.6.5 software. For nuclear antigen staining, Foxp3 kit from eBioscience (Germany) was used. For cytokine staining, 3–10 × 10^6 of cultured or *ex vivo* isolated cells were stimulated for 4–5 h with phorbol myristate acetate (PMA; 50 ng/ml) and ionomycin (ION; 1 μg/ml) both from Sigma (Germany) in T cell medium (RPMI-1640, 10% fetal calf serum, antibiotics, 55 μM β-mercaptoethanol) in the presence of Brefeldin A (eBioscience) at 37^{0}C. After the stimulation, cells were fixed with IC fixation buffer (eBioscience) and permeabilized with 0.5% saponin in FACS buffer. Fixable live stain dye Zombie Aqua kit (BioLegend, USA) was used to discriminate dead cells. CFSE (carboxyfluorescein diacetate succinimidyl ester; eBioscience) was used for cell proliferation analysis at a final concentration of 5 μM. The detailed list of antibodies is provided under Supplementary information.

2.4. In vitro suppression assay and iTreg induction

CD4 + CD45.1-CD62L + CD44- donor-derived naive T cells were sorted from chimeras and cultured for 72 h with coat-palted aCD3/CD28 (2ug each) either the T cell medium alone or in the presence of 50 ng/ml IL-2 and 5 ng/ml TGFβ. Cells were subsequently stained for surface markers and Foxp3 using Foxp3 kit. For the suppression assay naive CD4 + CD45.1 + CD62L + CD44-CD25- T cells and CD11c + dendritic cells were sort-purified from B6 wild type mice. Regulatory CD4 + CD25 + + CD45.1- cells were sorted from spleen and lymph nodes of wt and p100$^{-/-}$ chimeras. CFSE-labeled naïve T cells were cultured together with dendritic cells (10:1 ratio) and 1 μg/ml soluble αCD3 for 72 h at various ratios of wt or p100$^{-/-}$ Tregs.

2.5. Mouse necropsy and histology

Mice were sacrificed with CO_2 asphyxiation or by cervical dislocation. Organs were dissected and fixed in excess amount of 4% formalin (prepared from 37% formaldehyde with PBS) or 4% paraformaldehyde. Intestine and colon were flushed with PBS and lungs were perfused with formalin through trachea prior to fixation. Fixed tissues were embedded in paraffin and cut in 5 μm sections. H&E staining was performed according to the manufacturer'sprotocol (Sigma-Aldrich).

2.6. Autoantibodies staining

Liver and lung tissue from naïve Rag1$^{-/-}$ mice was homogenized in RIPA lysis buffer and 10 μg of protein were separated on 10% SDS gel and blotted onto Immobilon P membranes (Millipore). Membranes were blocked with 5% non-fat dry milk for 2 h and incubated with sera from individual mice at 1:200 dilution in 5% milk-TBST overnight. Membrane was washed with TBST, incubated with anti-mouse IgG-HRP and developed using ECL system (GE Healthcare).

2.7. Cytokine measurement in plasma

Blood was collected from anesthetized mice *via* retro-orbital bleeding and was allowed to coagulate at 4^{0}C. Blood plasma was obtained by centrifuging the samples at 10,000 g. Cytokines were measured using the Cytometric Bead Array (BD Biosciences).

2.8. Statistical analysis

Results are presented as the mean ± SD. Statistical significance was calculated using two-tailed, unpaired Student's *t*-test in Microsoft Excel 2003 software, unless stated otherwise. Survival was analyzed using Kaplan-Meyer plot in Graph Pad prism 5.0.1. *P* values less than 0.05 were considered significant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Plots represent one of the three or more comparable experiments.

3. Results

3.1. Lack of p100 causes homeostatic defect of T regulatory cells

As described previously, p100$^{-/-}$ mice show lethal phenotype as early as 3 weeks after birth, demonstrating both developmental and inflammatory phenotype (Ishikawa et al., 1997; Krljanac et al., 2014a; Koliesnik et al., 2017. To study the consequences of p100 deletion in hematopoetic cells we used a bone marrow transfer system, which allows us to analyze the bone marrow-derived cells, developed in a wild type environment. The CD4 compartment includes conventional T helper (Tconv) and regulatory T cells (Tregs) (Corthay, 2009). Our previous data showed decreased proliferation and disturbed homeostatic maintenance of peripheral p100$^{-/-}$ Tconv cells, but this alone cannot explain early postnatal lethality of the p100$^{-/-}$ mice (Ishikawa et al., 1997; Koliesnik et al., 2017). We hypothesized that the p100 mutation could also affect the cells controlling inflammation hence we decided to analyze the Treg compartment in greater detail. We used the intracellular Foxp3 staining to trace regulatory T cells *ex vivo* (Fontenot et al., 2003). Flow cytometrical analysis of lymphoid organs in bone marrow chimeras (BMC) revealed a significant decrease in the frequencies of Foxp3 + regulatory T cells in the spleen and peripheral lymph nodes of 100-/- BMC (Fig. 1a and 1b). The numbers of thymic Tregs were unchanged, which suggested a specific defect in peripheral Treg compartment. Next, staining of Foxp3 and Ki-67 in bone marrow chimeras revealed a twofold decrease in Ki-67 + cells among p100-deficient Tregs, which could account for a decreased Treg numbers (Fig. 1c and 1d). To confirm the cell intrinsic proliferation defect of p100$^{-/-}$ Tregs cells, we generated mixed bone marrow chimeras by co-transferring congenic wild type and p100$^{-/-}$ bone marrow into Ly5.1/Ly5.2 recipients in a 1:1 ratio. Under these competitive conditions

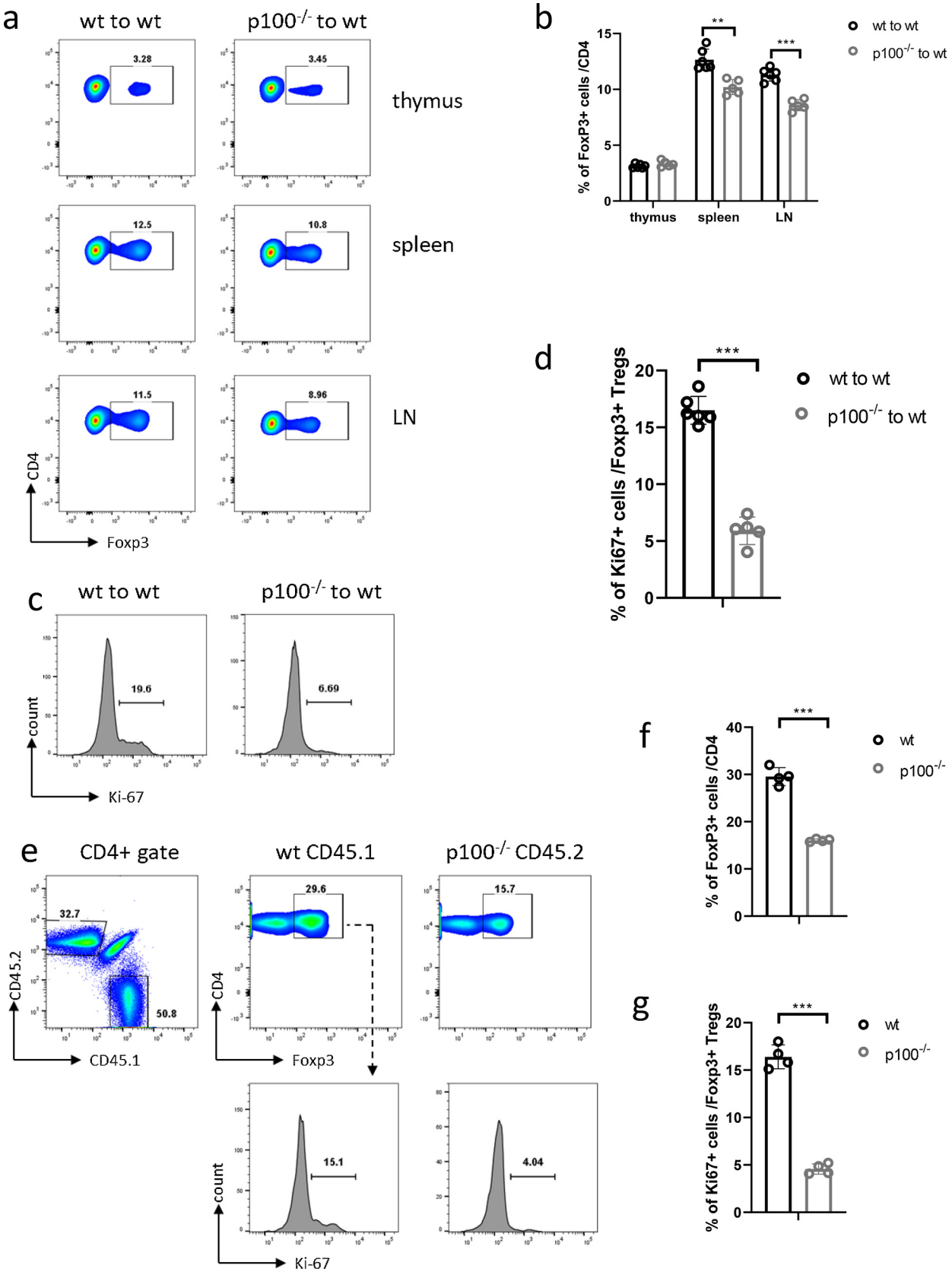

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Fig. 1. p100$^{-/-}$ Tregs have defective homeostasis.
a. Cells from thymus, spleen and lymph node of wt or p100-/- to Ly5.1 BMC were stained for Foxp3 and pregated on donor-derived CD45.1- CD4 + T cells.
b. Bar diagram is a representative of 4 independent experiments, with total of 20 animals per group.
c. Ki-67 staining of splenocytes from p100-/- or wt to wt BMC, pre-gated on single donor-derived CD4+ Foxp3+ Tregs.
d. Bar diagram summarizing Ki-67 expression in Tregs.
e. Competitive fitness of p100-/- Tconv and Tregs in mixed chimera, Splenocytes from mixed bone marrow chimera (CD45.1/CD45.2 were used as recipients) were stained for congenic markers and Ki-67. Histograms represent frequenices of Ki-67+ cells within the Treg gate for each of the genotype.
f. Tregs frequencies in the mixed bone marrow chimeras.
g. Proliferation of Tregs in mixed bone marrow chimeras *in vivo*. Data are representative for 10 mixed bone chimeras and shown as mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

mutant Tregs demonstrated a more pronounced homeostatic defect. Frequencies of Tregs among the p100$^{-/-}$ T cells were reduced twofold compared to control cells, but more severe than in single chimeras (Fig. 1e and 1f). In addition, steady state proliferation measured by Ki-67 showed a nearly threefold reduced numbers of proliferation mutant Tregs (Fig. 1e and 1 g). Therefore, we conclude that p100 deficiency negatively affects Treg homeostasis and "fitness". This defect is partially masked in the single chimeras, but revealed in a competitive bone marrow transfer.

3.2. Loss of p100 changes immunomodulatory phenotype of Treg cells

Tregs are characterized by the presence of a unique signature of phenotypical markers, which are crucial for suppressing unwanted inflammation in different organs. These markers include CD25 (alpha chain of IL-2 receptor), homing molecules like CD103, ATPases like CD39, regulators of T cell activation like CTLA-4, as well as activation markers including CD44 (Liston and Gray, 2014). In order to get a detailed picture of the p100$^{-/-}$ Treg phenotype, we evaluated some known Treg markers in p100$^{-/-}$ Tregs. We observed a strong downregulation of CD44 and CTLA-4 in Tregs from p100$^{-/-}$ BMC mice (Fig. 2a and b). Similarly, CD39 and CD73 were also significantly downregulated, with CD39 being reduced twofold and CD73 only marginally. KLRG-1 was virtually absent on mutant Treg cells. On the contrary, CD25 and GITR were slightly up regulated in p100$^{-/-}$ Tregs. We did not find a significant difference in the expression of ICOS CD103, Neuropilin and Helios. Furthermore, the same phenotype was observed in the mixed bone marrow chimeras indicating that it is intrinsic to p100$^{-/-}$ Tregs (Fig. 2c and Supplementary Fig. S1d). Our data show that, in addition to a proliferation defect, p100$^{-/-}$ Tregs have an aberrant phenotype. Therefore, loss of p100 leads to a reduction in mature Tregs, which lack activation markers.

3.3. p100$^{-/-}$ Tregs are dysfunctional in vitro

Considering, that the majority of the downregulated markers are associated with activation and suppressive capacity of regulatory T cells, we hypothesized that Tregs might also be defective in their function. Hence, we tested the capacity of Treg cells to suppress T cell activation *in vitro*. For this, sort-purified CD4+CD25+ cells from wt and p100$^{-/-}$ BMC were co-cultured with naive T cells. Indeed, wild type Treg cells efficiently suppressed responder T cell proliferation at a 1:1 Treg/Tresp ratio, compared to T cells without Tregs. On the contrary, p100-deficient Tregs were not able to suppress the proliferation of T responder cells at various ratios as effectively as wild type Tregs (Fig. 3a and b). Naïve T cells cultured with p100$^{-/-}$ Tregs proliferated almost as efficiently as under control conditions. Of note, frequencies of Foxp3+ cells among CD4+CD25+ cells were comparable between the genotypes (not shown). We also tested ability of p100$^{-/-}$ T cells to convert into induced Tregs in the presence of TGFβ and IL-2 *in vitro*. Compared to control cells mutant T cells developed significantly fewer Foxp3+ cells (Fig. 3c and 3d). Thus, we conclude that overt alternative NF-κB signaling disturbs Treg suppressive ability *in vitro* but has a rather minimal effect on iTregs induction.

3.4. p100$^{-/-}$ to Rag1$^{-/-}$ bone marrow chimeras develop spontaneous wasting disease and premature death

Bone marrow chimeras contain a significant portion of T cells of host origin. Strikingly, up to 30% of host T cells expressed Foxp3+ (not shown). This suggested that the host Treg cells are radioresistant and that they possibly compensate for the defective Treg population in p100−/− to Ly5.1 mice. To account for a possible compensatory effect of radioresistant T cells, we generated bone marrow chimeras with Rag1$^{-/-}$ mice as recipients. Strikingly, p100−/− to Rag1$^{-/-}$ mice started dramatically losing weight one month after transplantation (Fig. 4a), reaching up to 70% of their original weight by 1.5 months. General examination showed erect fur, hunched posture, inflamed eyelids and loose stool in p100$^{-/-}$ to Rag1$^{-/-}$ mice. They also had an increased mortality rate between the first and third month, and only few mice survived thereafter (Fig. 4b). Histological analysis of the mice revealed massive inflammatory lesions in various organs, among which liver and lungs displayed the strongest infiltrations (Fig. 4c). Lungs from p100−/− to Rag1$^{-/-}$ mice had severe thickening of bronchial ducts and signs of airway remodeling. Inflammation in the lungs also led to fibrosis and accumulation of collagen. Lung inflammation resembled macrophage alveolitis. FACS analysis of peripheral T cells showed increased proportion of activated CD4 cells in spleen of Rag1$^{-/-}$ BMC (Fig. 4d). Serum from p100−/− to Rag1$^{-/-}$ also contained an increased number of autoantibodies (Fig. 4e). Analysis of the cytokine profile of peripheral T cells revealed a predominant Th1 phenotype of p100−/− T cells, with 2–3 times more IFNγ producing CD4 cells (Fig. 4 f and Supplementary Fig. S1a), while IL-17 producing T cells were absent in p100−/− chimeras. To get a detailed view of the cytokine pattern, we measured a panel of various cytokines in the serum of Rag1$^{-/-}$ chimeras. While we could not detect any IFNγ, IL-6 or TNFα in the serum of control mice, these cytokines were up-regulated in p100−/− to Rag1$^{-/-}$ mice (Fig. 4g), TNFα was increased in all the samples measured (7/7) and MCP-1 (CCL2) was also significantly higher. However, in addition to autoimmune manifestations, p100−/− to Rag1$^{-/-}$ mice developed severe lymphopenia (Supplementary Fig. S1b), Total cellularity of spleen and lymph nodes was reduced more than 5-fold. Thus, although the proportion of activated, and possibly pathogenic memory cells was increased in mutant chimeras, their total numbers were strongly decreased. Therefore, our data suggest that in the presence of p100$^{-/-}$ hematopoetic compartment Rag1$^{-/-}$ mice develop systemic autoimmunity with increased IFNγ-producing T cells, Th1 cytokines in the serum and lymphopenia. Interestingly, lymphopenia was not observed in p100$^{-/-}$ to wt chimeras. This also demonstrates that only in the absence of host wild type lymphocytes p100-deficient bone marrow causes global autoimmune inflammation.

3.5. Adoptive transfer of wild type Tregs prevents the onset of wasting disease in p100$^{-/-}$ to Rag1$^{-/-}$ mice

Wasting disease observed in p100$^{-/-}$ to Rag1$^{-/-}$ can be caused by the inability of mutant Tregs to control spontaneous inflammation, the inability of mutant effector T cells to be suppressed by Tregs or a combination of both. To clarify this, we performed a rescue experiment in which we adoptively transferred purified wild type CD4+CD25+

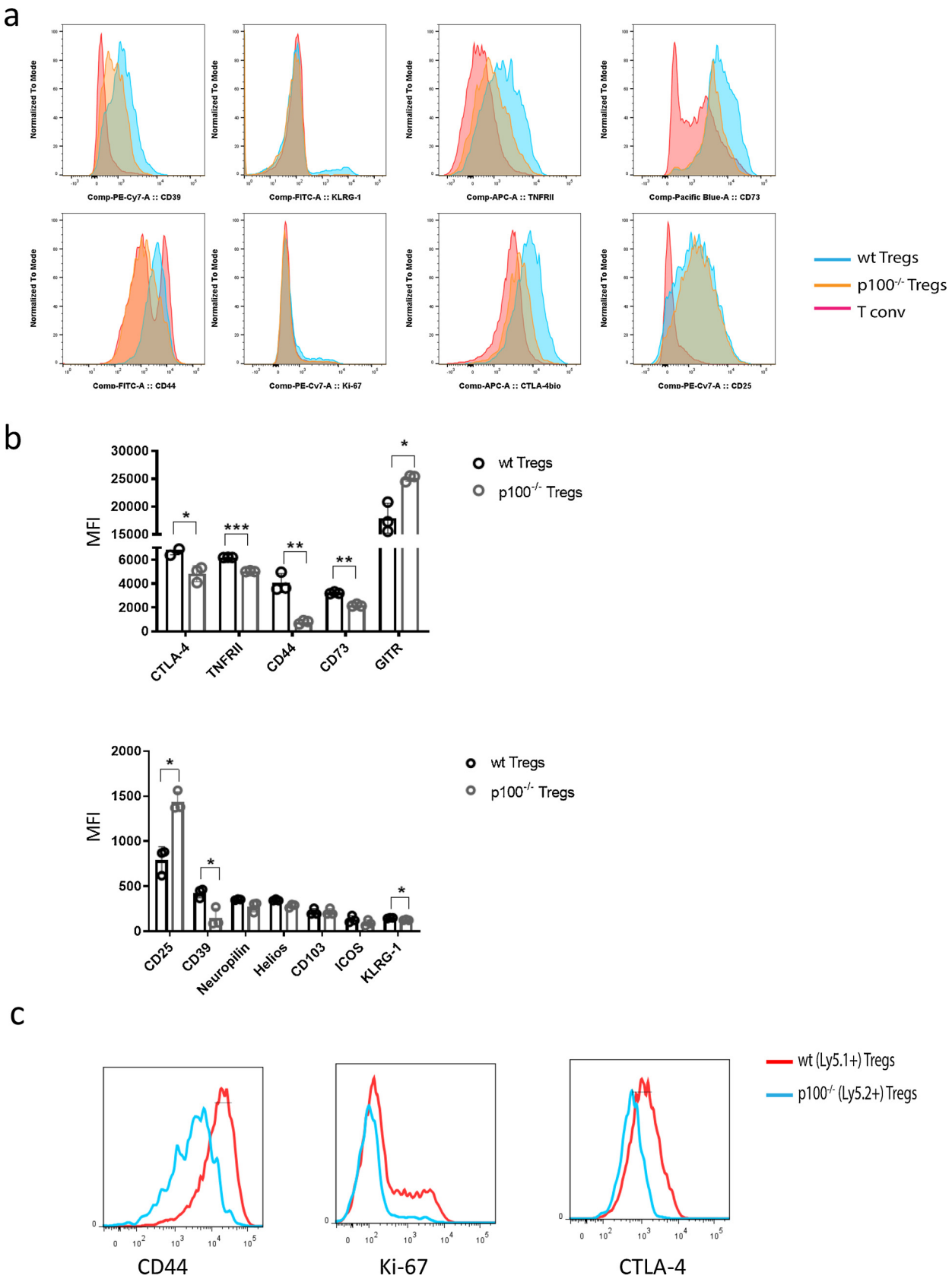

Fig. 2. p100$^{-/-}$ Tregs have an aberrant marker expression.

a. Representative flow cytometry histograms with an overlay of wt and p100-/- Treg markers. Conventional T cell staining is used as a control.

b. Summary of the phenotypic Treg marker expressed as an average MFI (mean fluorescence intensity) and depicted as a heat plot.

c. Assessment of Treg-associated surface and intracellular markers in mixed bone marrow chimeras wt (Ly5.1) or p100-/- (Ly5.2) CD4 + Foxp3 + cells. Data calculated from 3 independent experiments with 3–4 mice per group each. Representative of 15 bone marrow chimeras in each group, mean ± SD.* P < 0.05, ** P < 0.01, *** P < 0.001.

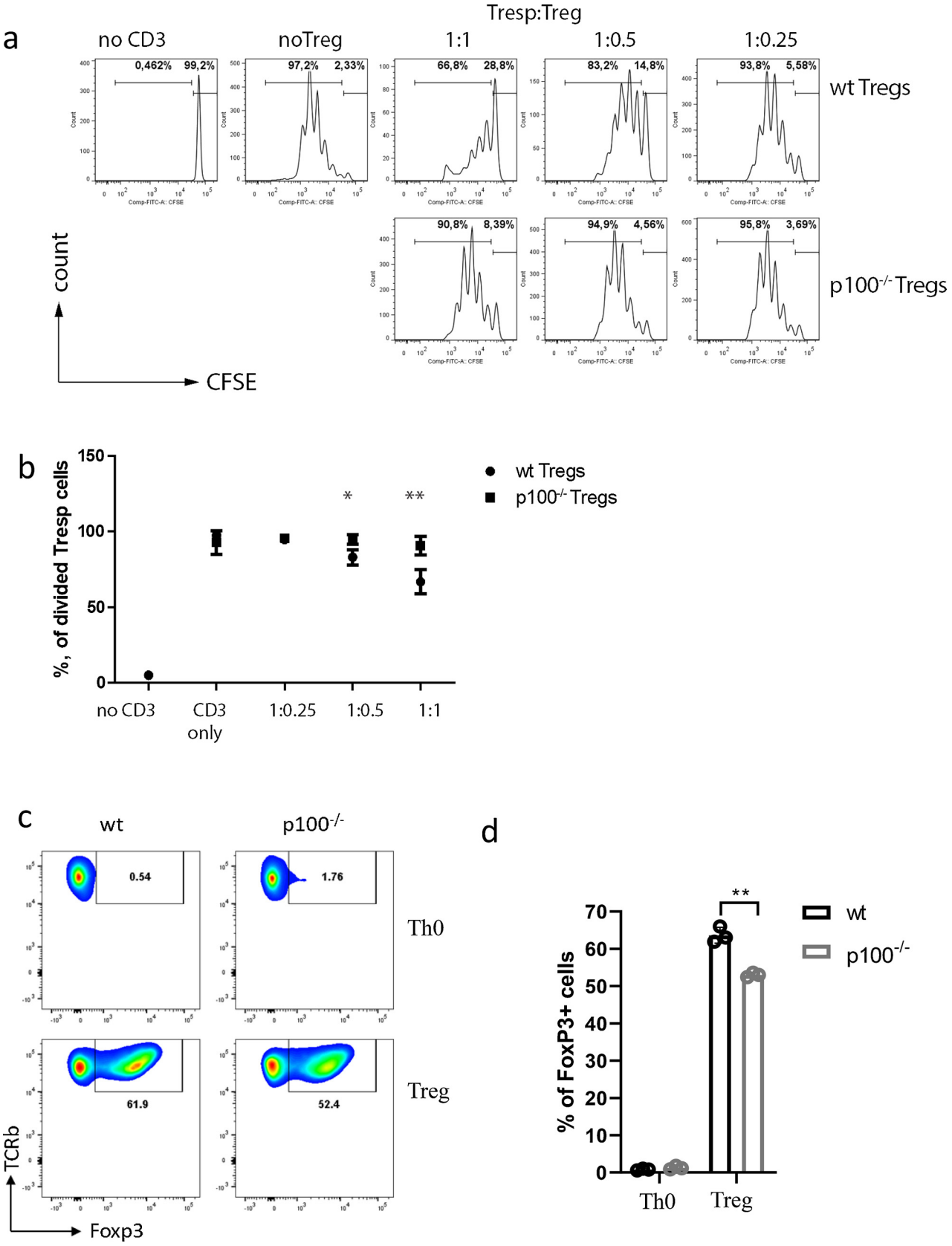

Fig. 3. p100$^{-/-}$ Tregs are dysfunctional *in vitro*.

a. FACS plots of the suppression assay setup. Naïve T cells (CD4 + CD44-CD62L+) were sorted from Ly5.1 or Ly5.2 B6 mice, stained with CFSE and cultured for 60–72 h with CD4 + CD45.1-CD25+ T (T responders) cells from wt or p100-/- to wt BMC in different ratios in the presence of CD11c + DC and 1 μg of anti CD3. Right panel indicates frequencies of Foxp3 cells among CD4 + CD25+ T cells.

b. Suppression assay. Representative of 3 independent experiments.

c. and d. Naïve T cells from from wt or p100-/- to wt BMC were cultured in the presence of anti CD3/CD28 only or plus IL-2 and TGFβ for 60 h. Representative data from 2 experiments, with pooled Tregs from 3 BMC each.

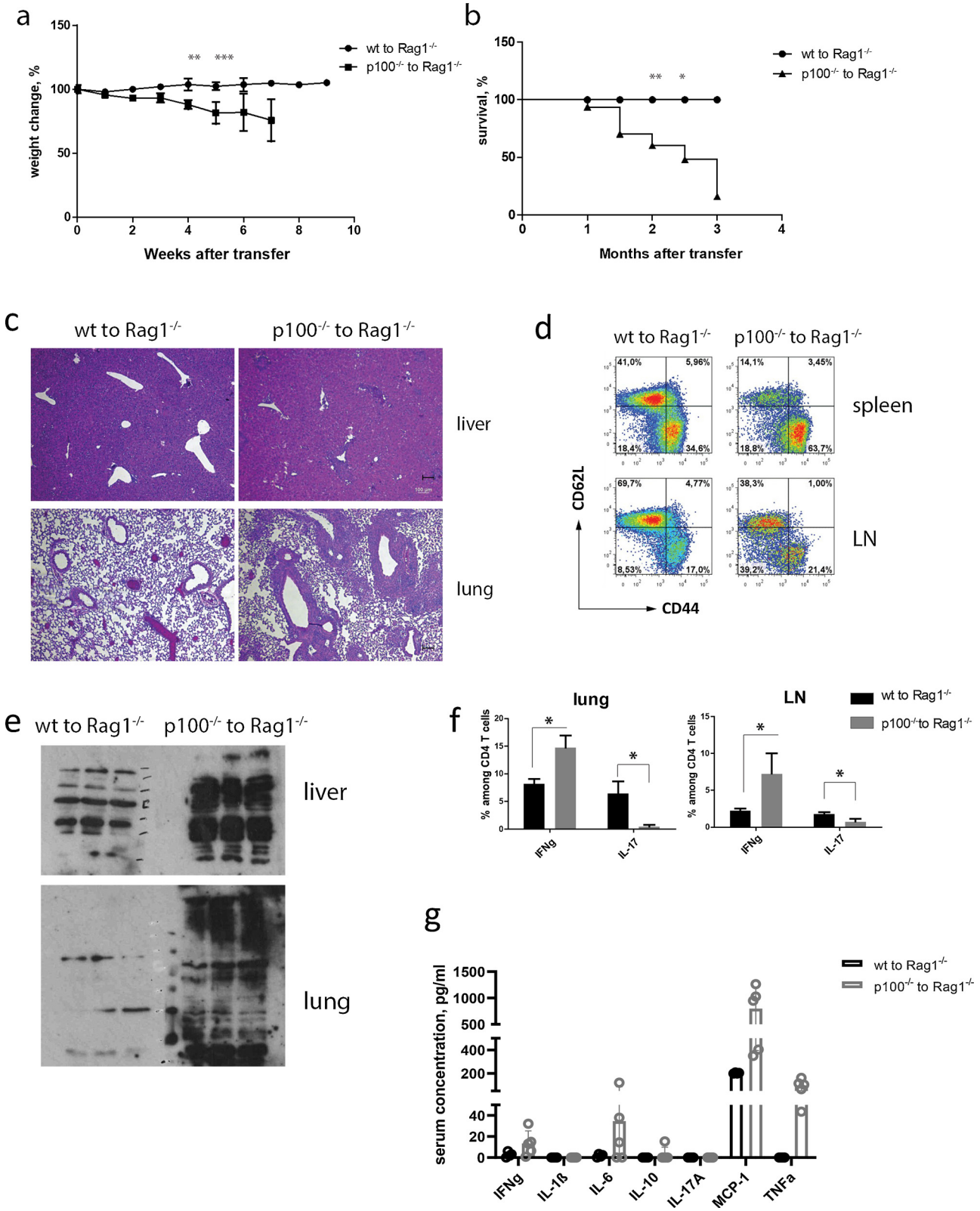

Fig. 4. p100$^{-/-}$ bone marrow chimeras develop lethal multiorgan inflammation in the absence of host lymphocytes.

a. Body weight curve of wt or p100-/- to Rag1-/- chimeras after bone marrow transfer.

b. Kaplan-Meyer plot depicting survival of Rag1-/- chimeras after transfer. Moribund mice were sacrificed and considered as dead.

c. H&E staining of Rag1-/- chimeras 1.5 months after transfer. Scalebar represents 100 μm.

d. CD44 and CD621 expression on CD4 T cells from Rag1-/- chimeras.

e. Immunoblot of liver and lung lysates from Rag1-/- mouse probed with serum from wt or p100-/- to Rag1-/- chimeras for autoantibody detection.

f. Cytokine analysis of CD4 T helper cells from Rag1-/- chimeras.

g. Serum cytokine concentration from Rag1-/- chimeras determined by cytometric bead array from the serum of 4 wt or 7 p100-/- to Rag1-/- mice.

For all experiments error bars indicate mean ± SD.; n.s.- not significant.* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

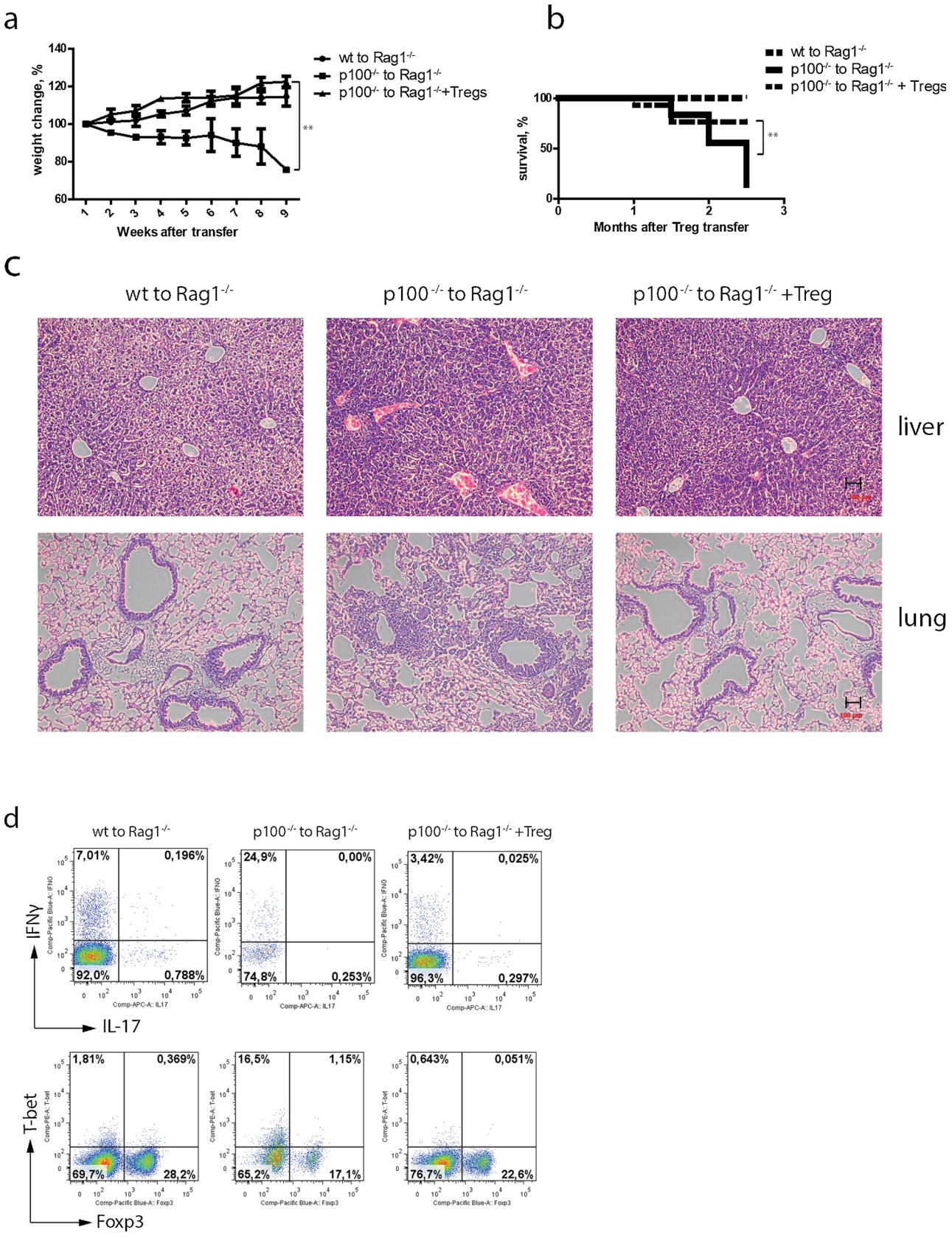

Fig. 5. Wild type Tregs can rescue the autoimmunity of p100$^{-/-}$ to Rag1$^{-/-}$ mice.

a. Kaplan-Meyer plot depicting survival of Rag1$^{-/-}$ chimeras after Treg transfer. Data from 2 independent experiments with 3 mice per group.

b. Body weight change with or without Treg transfer.

c. Hematoxylin&eosin staining of paraffin sections. Scalebar is 100 μm.

d. Cytokine and TF staining of splenic CD4 T cells in chimeras of respective genotype. Gated on TCRb + CD4 + CD45.1- (transferred Tregs) cells.

All data is from 2 independent experiments with 3–4 mice per group.

cells from Ly5.1 mice into p100$^{-/-}$ to Rag1$^{-/-}$ mice, three weeks after the bone marrow transfer. Interestingly, transfer of 1×10^6 Tregs was sufficient to significantly reverse the weight loss and mortality of p100$^{-/-}$ to Rag1$^{-/-}$ chimeras (Fig. 5a and b). Occurrence of lymphocytic infiltrations in liver and lung was also rescued (Fig. 5c). Transferred wild type Tregs also partially restored spleen and lymph node cellularity (Supplementary Fig. S2b). Regulatory T cell transfer also corrected IFNγ and T-bet expression by p100$^{-/-}$ T cells (Fig. 5d and Supplementary Fig. S2a). Collectively, we conclude that the observed wasting disease of p100$^{-/-}$ to Rag1$^{-/-}$ is caused by defective Treg cells and that p100$^{-/-}$ conventional T cells can be efficiently suppressed by wild type Tregs. Overall, our data suggest that unrestricted alternative NF-kappaB signaling in Tregs impairs their peripheral homeostasis, phenotype and suppressive function leading to a fatal autoimmunity in a lymphocyte-deficient context.

4. Discussion

Regulatory Foxp3-expressing T cells control immune reactions and prevent autoimmunity during the whole life of an organism (Kim et al., 2007). Interestingly, regulatory T cells in p100$^{-/-}$ BMC showed a cell-intrinsic homeostatic defect and functional defect. These Tregs had decreased CD44 expression, exhibiting a naive-like phenotype. This suggests that p100$^{-/-}$ Tregs as well as conventional T cells in general have an activation problem. Since effector CD44+ comprise a larger fraction of cells among Tregs with phenotype, it seems plausible that they are more sensitive to the mutation than conventional T cells. Indeed, it was shown that CD44 expression on Tregs positively correlates with their suppressive capacity *in vivo* (Liu et al., 2009). Similar to CD44, KLRG-1 expression that marks terminally differentiated Tregs was almost undetectable on p100$^{-/-}$ Tregs, which also indicates their low activation status (Podack et al., 2017). TNFRII is another marker, whose expression was curbed upon the loss of p100. Recently, signaling *via* TNFRII was shown to drive Treg induction in the thymus by modulating TCR strength and sensitivity to IL-2 (Mahmud et al., 2014). TNFRII$^{-/-}$ thymus-derived Tregs were not able to suppress naïve T cell-induced colitis in Rag1$^{-/-}$ mice (Housley et al., 2011). We did not observe, however, a decrease in GITR + cells among p100$^{-/-}$ Tregs, which points to a selective manner of the Treg phenotype. Interestingly, expression of CD25 is slightly up-regulated on p100$^{-/-}$ Tregs, arguing against the full anergy of these cells. Tregs from p100$^{-/-}$ to wt BMC possessed significantly decreased suppressive activity than wild type cells. One explanation for the decreased suppressive activity is the changed ratio of effector/naïve-like cells among Treg fraction in mutant chimeras. Since p100$^{-/-}$ Treg fraction contains more naïve-like cells they could be less suppressive than CD44+ fraction. To rule this out, further experiments dissecting suppressive ability of CD62L + versus CD44+ p100$^{-/-}$ Treg populations are needed. p100$^{-/-}$ Tregs display a complex phenotype with several downregulated markers. Therefore, it is not clear whether a single marker or a combination of them is responsible for the suppression defect. Functional evaluation of separate sub-populations is required further to clarify this. It was noteworthy that wild-type mice reconstituted with p100$^{-/-}$ bone marrow occasionally exhibited mild colitis and reduced weight, but seemed healthy otherwise. This could be explained by additional effect of the mutation on conventional T cells that are known to have an activation defect and a block in Th17 differentiation (Koliesnik et al., 2017). Since T cells are more radioresistant than B and myeloid cells, host Tregs could compensate the loss of function in p100$^{-/-}$ Tregs (Komatsu and Hori, 2007). To test this possibility and to overcome the presence of radioresistant T cells we used Rag1$^{-/-}$ recipients for chimera generation. In the absence of host T cells Rag1-deficient mice developed a strong multiorgan inflammation after receiving the p100$^{-/-}$ bone marrow. This inflammation had characteristics of autoimmune disease but in addition was accompanied by severe reduction of peripheral lymphocytes. In agreement with our findings on autoimmune potential of p100$^{-/-}$ T cells, another group reported the development of lethal inflammation in mice with transgenic overexpression of NIK in T cells (Murray et al., 2011). NIK abundance made Tregs less suppressive and rendered T conventional cells more resistance to Tregs. Although, we cannot exclude that in our model T cells are also less sensitive to Treg action, Treg transfer was sufficient to rescue all signs of inflammation. Additionally, Rag1$^{-/-}$ mice with mixed bone marrows do not develop inflammatory pathologies, indicating that the presence of wild type cells can prevent the disease manifestation (not shown). Similar to our observations it was reported that overexpression of p52 in T cells does not predispose mice to tumors, but rather to autoimmunity (Wang et al., 2008). Another group showed that overexpression of p52 combined with concomitant deletion of *nfkb2* in lymphocytes led to fatal lung inflammation in mice (Yang et al., 2010). This was assumingly caused by autoreactive Th1 cells and activated alveolar macrophages. However, in both abovementioned studies the authors did not discriminate between pathogenic T cells and Tregs. It is likely that Tregs, affected by the described mutations, also contributed to the disease outcome. Our results suggest that the multiorgan inflammation in Rag1$^{-/-}$ recipients is caused by autoreactive T cells. These T cells have impaired homeostasis, activation disadvantage and defective Th17 lineage development (Koliesnik et al., 2017). However, even with such defective T cells the absence of functional Tregs tips the balance towards inflammation and leads to autoimmune disease.

Adoptive transfer of thymus-derived Tregs (tTregs) was shown to rescue lethal inflammation of *scurfy* mice, which carry a truncated form of the Foxp3 protein (Fontenot et al., 2003). Another report suggested that T cells differentiated *in vitro* with polyclonal stimulation and TGFβ are also able to suppress inflammation in *scurfy* mice (Huter et al., 2008). This demonstrates that the presence of functional Treg cells independent of their origin can prevent disease development and that Foxp3 is needed only within this fraction of T cells. Combined action of Tregs from thymus and those induced in the periphery is required to protect the host from excessive inflammation. Rag1$^{-/-}$ mice reconstituted with p100$^{-/-}$ bone marrow display complex inflammation at multiple sites and produce autoantibodies suggesting a defect in both thymic-derived and induced Tregs. A portion of wild-type CD4+CD25+ cells was able to prevent the inflammation onset in these mice and extend their survival beyond 3 months after the transfer. While the inoculate consists of both types of Tregs, it can also contain some conventional T cells, whose conversion into Treg cells can be an important mechanism against autoimmunity [55]. The rescue effect also depends on the time and number of Tregs transferred. We used transferred Treg injection mice two weeks after bone marrow transplantation, however if mice already develop signs of inflammation, their body weight and survival were only partially rescued. Similarly, mice that obtained 0.4×10^6 Tregs gained more weight, but still developed mild inflammation (not shown). We assume that the Treg pool for the transfer has to be diverse enough to cover many self-antigens and that once inflammation has started Tregs become less efficient. Treg transfer also corrects IFNγ and T-bet expression in conventional T cells of p100$^{-/-}$ to Rag1$^{-/-}$ mice. This points to Th1 inflammation as a main cause of their wasting phenotype. Moreover, Tregs also partially rescue lymphopenia by increasing the numbers of T and B cells in spleen and lymph nodes, indicating that loss of lymphocytes could also be due to their increased migration to the tissues. Recently another group has shown that upon deletion of *nfkb2* in regulatory T cells mice develop autoimmunity (Grinberg-Bleyer et al., 2018). This group provided evidence that in the absence of p100 RelB can translocate into the nucleus upon TCR stimulation and activates the pro-inflammatory gene signature.(Grinberg-Bleyer et al., 2018) This is in agreement with our observation that p100 C-terminus acts as a negative regulator of RelB activation (Lovas et al., 2012); Krljanac et al., 2014b; Koliesnik et al., 2018. However, in our study we observed a decreased homeostasis in Tregs combined with an autoimmune lymphopenia that was not observed by Grinberg-Bleyer and colleagues. Our mouse model carried a

truncated form of p100 with a functional p52. Since nfkb2 is a target gene of p52/RelB (Ishikawa et al., 1997); (Lovas et al., 2012), it is possible that p100 truncation leads to a postivie feedback loop of RelB signaling in Tregs which results in a more pronounced Treg defect and lymphopenia.

It is puzzling how lymphopenia contributes to the progression of the disease in $Rag1^{-/-}$ chimeras. Lymphopenia has been linked to autoimmune diseases in several studies (Schulze-Koops, 2004). One report suggested that an "emptiness" of a T cell niche drives the proliferation of short-lived memory T cells, which precipitate autoimmunity (King et al., 2004). Moltedo and colleagues reported that depletion of Tregs in $Foxp3^{DTR}$ mouse leads to acute but transient T cell lymphopenia, which precedes autoimmunity (Moltedo et al., 2014). It is plausible therefore that a lymphopenic environment of $Rag1^{-/-}$ mice favors the accumulation of the rapidly dying memory T cells that are not efficiently suppressed by the regulatory T cells. Overall, we conclude that unrestricted alternative NF-κB affects homeostasis and suppressive ability of T regulatory cells. These defects are masked by residual Tregs in wild type mice but lead to an unleashed inflammation in $Rag1^{-/-}$ recipients. Conceptually, it appears that signaling *via* TNFR superfamily puts a brake on Tregs which is necessary for the efficient immune response and cancer clearance (Murray et al., 2011). Our data provide additional evidence on the p52-RelB axis in T cell biology and may be considered as a therapeutic target for Treg manipulation in tumors.

Conflict of interest

The authors declare no financial or commercial conflict of interest

Author declaration

We declare here that there are no known conflict of interest associated with this manuscript and there is no financial support that could have influenced its outcome. We confirm that the manuscript has been read and approved by all the authors. We also confirm that the order of authors listed in the manuscript has been approved by all of us.

We declare that any aspect of the work covered in this manuscript that involved animal experiments has been conducted with the approval of all relevant bodies and that such approvals are acknowledged in the manuscript. We understand that the Corresponding author is the sole contact for the Editorial process. He is responsible for the communication with the other authors about the progress, submissions of revisions and final proofs. We confirm that we have provided a correct, current email address which is accessible by the Corresponding Author and configured to accept email from (ev.kolesnik@gmail.com). Signed by all the authors as follows:

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.imbio.2019.06.001

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