

Reduced CD18 Levels Drive Regulatory T Cell Conversion into Th17 Cells in the CD18^{hypo} PL/J Mouse Model of Psoriasis

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Defective development and function of CD4⁺CD25^{high} Foxp3⁺ regulatory T cells (Tregs) contribute to the pathogenesis of psoriasis and other autoimmune diseases. Little is known about the influence of adhesions molecules on the differentiation of Foxp3⁺ Tregs into proinflammatory Th17 cells occurring in lesional skin and blood of psoriasis patients. In the CD18^{hypo} PL/J mouse model of psoriasis, reduced expression of CD18/β₂ integrin to 2–16% of wild-type levels is associated with progressive loss of Tregs, impaired cell–cell contact between Tregs and dendritic cells (DCs), as well as Treg dysfunction as reported earlier. In the present investigation, Tregs derived from CD18^{hypo} PL/J mice were analyzed for their propensity to differentiate into IL-17–producing Th17 cells in vivo and in in vitro Treg–DC cocultures. Adoptively transferred CD18^{hypo} PL/J Tregs were more inclined toward conversion into IL-17–producing Th17 cells in vivo in an inflammatory as well as noninflammatory environment compared with CD18^{wt} PL/J Tregs. Addition of neutralizing Ab against CD18 to Treg–DC cocultures in vitro promoted conversion of CD18^{hypo} PL/J Tregs to Th17 cells in a dose-dependent manner similar to conversion rates of CD18^{hypo} PL/J Tregs. Reduced thymic output of naturally occurring Tregs and peripheral conversion of Tregs into Th17 cells therefore both contribute to the loss of Tregs and the psoriasiform dermatitis observed in CD18^{hypo} PL/J mice. Our data overall indicate that CD18 expression levels impact Treg development as well as Treg plasticity and that differentiation of Tregs into IL-17–producing Th17 cells is distinctly facilitated by a subtotal deficiency of CD18. *The Journal of Immunology*, 2013, 190: 000–000.

Psoriasis, a common autoimmune disease with a prevalence of 2–3% in the worldwide population depicting with characteristic erythematous skin lesions covered with white silvery scales, is associated with severely reduced quality of life (1). Ten to 40% of psoriasis patients develop a destructive arthritis.

Abnormal function of T lymphocytes is considered a major cause of psoriasis (2–4). Dysfunctional regulatory T cells (Tregs) contribute to the unrestrained generation of pathogenic T cells in psoriasis in humans and in the CD18^{hypo} PL/J mouse model as reported earlier (5–7). CD4⁺CD25^{high} Foxp3⁺ naturally occurring Tregs (nTregs) expressing transcription factor (TF) Foxp3, a master regulator of Treg development and function (8), are normally considered inhibitors of autoimmune responses (recently reviewed in Ref. 9). Under proinflammatory conditions, however, Tregs may differentiate into inflammation-associated Th17 cells, a paradigm shift with as yet largely unknown consequences for human disease initiation or progression (8, 9). Deficiency in CD4⁺CD25^{high} Foxp3⁺ Tregs mainly plays a role in acute exacerbation of psoriasis when the balance of immune suppression and immune activation is just about to shift, whereas disease maintenance occurs through amplification of inflammatory cytokines (7). Th17 cells, a subset of Th cells characterized by production of IL-17 and expression of TF retinoic acid–related orphan receptor (ROR) α and γ (10, 11), have been implicated in the pathogenesis of psoriasis and other autoimmune inflammatory diseases (12, 13). Although the primary function of Th17 cells is the clearance of pathogens that are not adequately handled by Th1 or Th2 cells, Th17 cells are also potent inducers of tissue inflammation due to production of effector cytokines such as IL-17, IL-21, and IL-22. Earlier work established IL-23 as a survival factor for Th17 cells and showed that in vitro differentiation of Th17 cells unexpectedly proceeds through a developmental pathway partially shared with the anti-inflammatory Foxp3⁺ Treg population (reviewed in Ref. 14).

We previously reported on the CD18 hypomorphic (CD18^{hypo}) PL/J mouse model of psoriasis with reduced expression of CD18 (β₂ integrin, Itgb2) to 2–16% of wild-type levels as a consequence of a hypomorphic gene mutation (15–18). CD18 represents the

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K.S. and M.G. designed and performed experiments and wrote the manuscript. T.P. and H.W. designed experiments and commented on the manuscript. L.B. designed and performed experiments. A.H. provided expert technical assistance with preparation of cryosections and immunostaining. A.S. designed and performed experiments. K.S.-K. designed experiments and wrote the manuscript.

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Abbreviations used in this article: CD18^{hypo} PL/J, PL/J mice with a hypomorphic mutation of the CD18 gene; DC, dendritic cell; DP, double-positive; iTreg, induced regulatory T cell; nTreg, naturally occurring regulatory T cell; ROR, retinoic acid–related orphan receptor; SP, single-positive; Teff, effector T cell; TF, transcription factor; Treg, regulatory T cell.

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common β -chain of four different heterodimeric integrins expressed on distinct hematopoietic cell subsets, namely CD18/CD11a, CD18/CD11b, CD18/CD11c, and CD18/CD11d, that as leukocyte adhesion molecules regulate cell–cell contacts through interaction with >20 known ligands, such as ligands of the ICAM family (19, 20). Homozygous CD18^{hypo} PL/J mice spontaneously develop a T cell–mediated psoriasiform skin disease at 12–14 wk of age that closely resembles human psoriasis histologically, clinically, and in its polygenic base (16, 21). Evidence indicating that reduced CD18 expression may also causally be involved in the development of psoriasis in humans comes from the clinical observation that some patients suffering from leukocyte adhesion deficiency syndrome 1, even with moderately reduced CD18 expression levels, develop a psoriasiform skin disease (22). Linkage analysis of psoriasis families has identified a susceptibility locus within a region on chromosome 17, which includes the ICAM-2 locus, an important ligand of the CD11/CD18 heterodimers (23).

According to recent studies, peripheral blood–derived Tregs from patients with severe psoriasis easily differentiate into a Th17-associated phenotype, a process promoted by IL-23 (24, 25). Previous data from our group (6) show that CD4⁺CD25⁺CD127[−] Treg numbers as well as their suppressive properties progressively decline in affected CD18^{hypo} PL/J mice. Interestingly, CD18^{wt} Tregs possess higher Foxp3 levels compared with CD18^{hypo} Tregs, suggesting that wild-type CD18 expression levels may be critical to maintain Treg properties (6). As nTregs from the thymus and peripherally induced Tregs (iTregs) are mandatory to suppress the development of autoimmune disorders, we have set out to study the role of CD18 in thymic nTreg numbers and in regulating the fate of peripheral Tregs. To investigate if conversion of Tregs into IL-17–producing Th17 cells contributes to the loss of peripheral CD4⁺CD25⁺CD127[−] Tregs in lymphatic organs and to the onset of psoriasiform dermatitis in CD18^{hypo} PL/J mice, we analyzed peripheral differentiation of CD18^{hypo} PL/J Tregs into Th17 cells in vitro and in vivo in greater detail. We found that altered nTreg development in thymi of young, still unaffected CD18^{hypo} PL/J mice and peripheral conversion of Tregs into Th17 cell both contribute to the loss of tolerance in CD18^{hypo} PL/J mice, ultimately resulting in the clinical manifestation of psoriasiform dermatitis. These findings further support the concept that CD18 heterodimeric molecules, through their role in mediating cell–cell contacts between immune cell subsets, are essential for proper development and function of nTregs, peripheral Treg maintenance, and the suppression of psoriasiform skin disease in PL/J mice.

Materials and Methods

Mice

PL/J mice with a hypomorphic mutation of the CD18 gene (CD18^{hypo}) have been described (8). CD18^{+/+} littermates (CD18^{wt}) resulting from heterozygous crosses served as wild-type controls. To evaluate the severity of the psoriasiform phenotype, an adapted psoriasis area and severity index score was determined for affected CD18^{hypo} PL/J mice used for experiments as described earlier (16). The modified psoriasis area and severity index score reflects the following clinical phenotypes: 0, no symptoms; 1, slight erythema of the ears; 2, strong erythema of the ears; 3, slight hair loss on the head; 4, extensive hair loss, including the trunk; 5, slight hair loss, isolated scaling; 6, extensive hair loss, isolated scaling; 7, extensive hair loss, widespread slight scaling; 8, moderate scaling of a large area of the body; 9, widespread hair loss, strong scaling of a few smaller areas; and 10, extensive hair loss, extensive scaling of a large area of the body. For all experiments, CD18^{hypo} PL/J mice displaying a strong psoriasiform phenotype were used. All mice were kept in the animal facility of the Tierforschungszentrum of the University of Ulm under pathogen-free conditions. All procedures were done in accordance with the guidelines for animal experimentation approved by the Regierungspräsidium Tübingen, Germany.

FACS analysis

Abs used for FACS analysis are as follows: PerCP-conjugated rat anti-mouse CD4 (clone RM4-5; BD Biosciences), PE-Cy7-conjugated anti-CD4 (clone-GK1.5; eBioscience), PE-conjugated rat anti-mouse CD25 (Miltenyi Biotec), eFluor 450-conjugated anti-mouse CD127 (clone-A7R34; eBioscience), Alexa Fluor 647-conjugated anti-Foxp3 (clone-MF23; BD Biosciences), FITC-conjugated anti-mouse IL-17A (clone eBio17B7; eBioscience), allophycocyanin-Cy7-conjugated IL-17A (clone-TC-11-18H10; BD Biosciences), Alexa Fluor 488-conjugated anti-Stat5 pY694 (BD Biosciences), and PerCP-conjugated ROR γ (t) (clone B2D; eBioscience). Cells isolated from spleens of CD18^{wt} PL/J and CD18^{hypo} PL/J mice were processed for FACS analysis as previously described (16). To monitor Foxp3 and IL-17 expression on mouse Tregs, Tregs cocultured with allogeneic dendritic cells (DCs) were first stained for surface markers with anti-CD4, anti-CD25, and anti-CD127 Abs on days 0, 3, and 7. After fixation and permeabilization, intracellular Foxp3 followed by IL-17A was detected using the mouse Foxp3 buffer Set (BD Pharmingen) according to the manufacturer's protocols. Isotype IgG was used as control for all experiments. All FACS analyses were performed using an FACSCalibur and FACSDiva (BD Biosciences) as well as FlowJo (Tree Star) software.

Immunofluorescence staining

Frozen cryosections (5 μ m) of mouse back skin and lymph nodes were fixed in ice-cold acetone for 10 min before staining. To detect T cell subpopulations, sections were first incubated with Ab against mouse CD4 (clone-L3T4; eBioscience) or Foxp3 (clone-FJK-16s; eBioscience). Alexa Fluor 488-conjugated anti-goat Ab (Molecular Probes) was used as a secondary Ab. Subsequently, anti-mouse IL-17 Ab (H-132; Santa Cruz Biotechnology) or anti-mouse ROR γ t (clone-Q31-378; BD Pharmingen) was added for the detection of IL-17–producing cells and ROR γ t as Th17-defining TF, respectively, followed by incubation with Alexa Fluor 555-conjugated secondary Abs (Molecular Probes). Isotype Ig served as negative control. DAPI (Sigma-Aldrich) was used to stain nuclei. All Abs were diluted in a 1% Ab diluent (DakoCytomation). Photomicrographs were produced using a Zeiss Axiophot microscope (Carl Zeiss) and corresponding software.

Treg and DC preparation

For in vitro experiments, CD4⁺CD25⁺ Tregs were isolated from spleens of CD18^{wt} PL/J or CD18^{hypo} PL/J mice using the CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (Miltenyi Biotec). Subsequently, cells were sorted for CD4⁺CD25⁺ CD127[−] Tregs with an FACS Aria II (BD Biosciences). For allogeneic stimulation of Tregs, DCs prepared from either BALB/c, CD18^{wt} PL/J, and CD18^{hypo} PL/J or CD18^{wt} C57BL/6J and CD18^{hypo} C57BL/6J mice were used as indicated in respective experiments. To generate immature DCs, bone marrow cells from femurs of mice from respective mouse lines were isolated and subsequently cultured in the presence of GM-CSF (20 ng/ml) and IL-4 for 7 d. Quality of bone marrow–derived DCs was assessed by analysis of expression of CD11b, CD11c, CD80, CD86, and MHC class II via FACS.

MLR

Tregs derived from CD18^{hypo} PL/J, CD18^{wt} PL/J CD18^{wt} C57BL/6J, and CD18^{hypo} C57BL/6J mice and bone marrow–derived allogeneic DCs (irradiated with 30 Gy) from BALB/c, CD18^{wt} PL/J, CD18^{hypo} PL/J, CD18^{wt} C57BL/6J, and CD18^{hypo} C57BL/6J mice were cocultured in U-bottom 96-well plates (Nunc) in the presence of 500 U/ml recombinant murine IL-2 (PeproTech) for 7 d. For CD18 blocking experiments, purified Tregs were cocultured with allogeneic DCs in the presence of 20 μ g/ml neutralizing Ab against murine CD18/ β 2 Integrin (clone GAME-46; BD Pharmingen) and isotope-matched IgG, with 500 U/ml recombinant murine IL-2 for 7 d. For dose-response experiments, neutralizing Ab against CD18 was added in different concentrations as 0, 5, 10 and 20 μ g/ml.

Adoptive transfers

Freshly isolated CD4⁺CD25⁺CD127[−] cells purified by MACS and FACS sorting as described above were labeled with CFSE (Molecular Probes) according to the manufacturer's protocol. A total of 1×10^6 FACS-sorted, CFSE-labeled CD4⁺CD25⁺CD127[−] Tregs from CD18^{wt} PL/J or CD18^{hypo} PL/J mice were transferred i.v. into recipient mice as described (6).

Statistical analysis

Where indicated, *p* values were calculated using a Student *t* test or one-way ANOVA and significance levels denoted as follows: **p* < 0.1, ***p* < 0.05, and ****p* < 0.01.

Results

Altered development of CD4⁺ nTregs in thymi of young unaffected CD18^{hypo} PL/J mice

As reported earlier, thymic involution is accelerated in CD18^{hypo} PL/J mice upon manifestation of inflammation and psoriasiform dermatitis (6). Whereas numbers and percentages of double-negative, double-positive (DP), and single-positive (SP) thymocyte subsets are grossly normal in PL/J CD18^{hypo} mice prior to the onset of skin disease, percentages of thymic Treg precursors and peripheral CD4⁺CD25⁺CD127⁻ Tregs progressively decline in affected mice (6). To systematically analyze the effect of reduced CD18 expression levels on the different stages of Treg development in the CD18^{hypo} PL/J mouse model independent from systemic inflammation, we first examined nTreg development in thymi of unaffected CD18^{hypo} PL/J mice prior to disease manifestation at an age between 4 and 6 wk. As a consequence of the reduced CD18 expression in CD18^{hypo} PL/J mice, the fraction of CD4⁺CD25⁺ thymocytes, representing potential nTreg precursors, was significantly reduced by ~50% compared with CD18^{wt} PL/J littermates (Fig. 1A). Although the fraction of total CD4⁺Foxp3⁺ nTregs was also reduced by ~50% in unaffected CD18^{hypo} PL/J thymi, Foxp3 expression of CD4⁺CD25^{high+} thymocytes was normal ranging from 40–55% Foxp3⁺ CD4⁺CD25^{high+} cells for both CD18^{hypo} or CD18^{wt} PL/J thymi (not shown). Our results therefore indicate defective upregulation of CD25a (IL-2R α) on CD18^{hypo} PL/J CD4⁺ SP thymocytes. Consistently, stimulation of thymocytes with IL-2 or a combination of IL-2 and anti-CD3 for 24 h in vitro resulted in reduced upregulation of CD25a on CD18^{hypo} PL/J CD4⁺ SP thymocytes and reduced overall Foxp3 induction compared with CD18^{wt} thymocytes (Fig. 1B). Phosphorylation of TF Stat5 (pY694), which mediates signaling via the IL-2R and regulates Foxp3 expression, was also reduced in CD4⁺ thymocytes derived from CD18^{hypo} compared with CD18^{wt} PL/J upon in vitro stimulation with IL-2 for 30 min as detected by intracellular cytokine staining and FACS analysis (Fig. 1C). mRNA expression analysis of Foxp3 in total thymocytes by real-time quantitative PCR confirmed reduced overall levels of Foxp3 in CD18^{hypo} PL/J thymi (data not shown). Because expression levels of the Th17-specific genes IL-17 and ROR γ t were only minimally increased in CD18^{hypo} PL/J thymocytes as detected by FACS analysis and quantitative PCR (Supplemental Fig. 1 and data not shown), we cannot conclude at this point that CD18^{hypo} PL/J thymic nTregs were already more prone to become Th17 cells or that nTh17 cell development was increased.

Infiltration of lesional skin of CD18^{hypo} PL/J mice with IL-17-producing CD4⁺ T cells and in situ conversion of Foxp3⁺ Tregs into IL-17⁺ T cells

To confirm the importance of Th17 cells in psoriasiform dermatitis of CD18^{hypo} PL/J mice, we then analyzed the composition of the cellular infiltrate in inflamed CD18^{hypo} PL/J skin in comparison with healthy skin of CD18^{wt} PL/J mice by immunofluorescence staining of skin sections. Lesional skin of CD18^{hypo} PL/J mice showed remarkably increased numbers of CD4⁺IL-17⁺ cells compared with healthy skin (Fig. 2A, 2B). Because our previous data indicated a progressive reduction of Treg numbers in CD18^{hypo} PL/J mice, we hypothesized that the increased numbers of CD4⁺IL-17⁺ cells in skin infiltrates of these mice may be a result of increased conversion of Tregs into Th17 cells. To verify the occurrence of this conversion process of Tregs in inflamed skin of affected CD18^{hypo} PL/J mice and detect potential transitional stages expressing genes typical for both subsets, Treg and Th17 cells, skin cryosections were first double-stained with labeled Abs

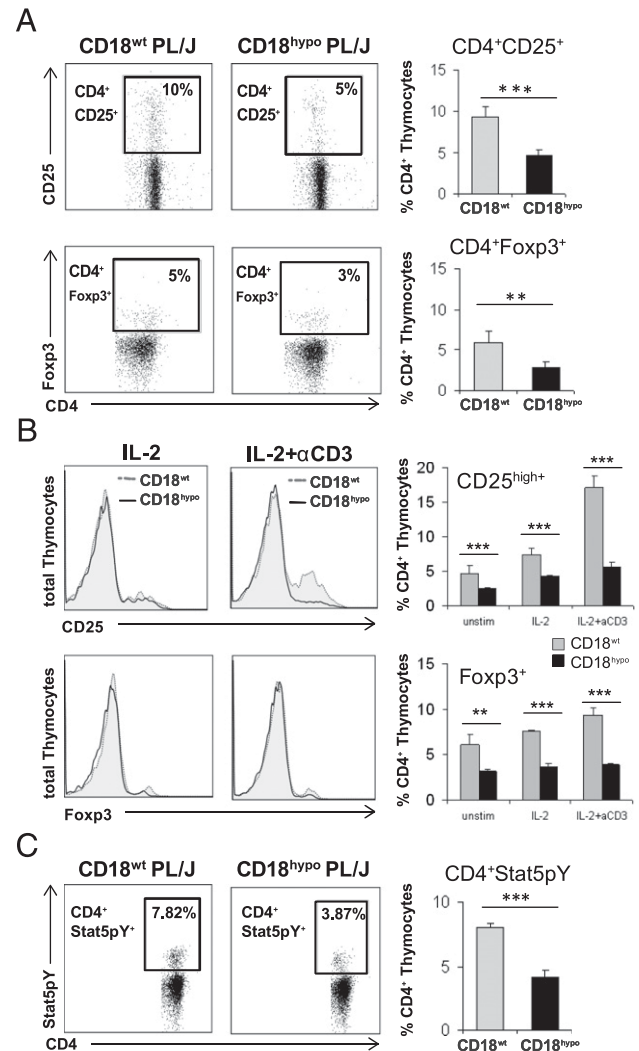


FIGURE 1. Altered development of CD4⁺ nTregs in thymi of young CD18^{hypo} PL/J mice. Thymocytes were prepared from 4–6-wk-old CD18^{hypo} and CD18^{wt} PL/J mice and analyzed by flow cytometry ex vivo or after stimulation as indicated. For FACS analyses showing nTreg development, we first gated on SP CD4⁺ thymocytes and then on CD4⁺CD25⁺ nTreg precursors. Two to three independent experiments with two to three mice of each genotype were performed; representative FACS plots are shown. **(A)** Frequencies of CD4⁺CD25⁺ nTreg precursors and CD4⁺Foxp3⁺ nTregs are significantly reduced in thymi of 4–6-wk-old CD18^{hypo} PL/J mice (black) compared with age-matched CD18^{wt} PL/J mice (gray); ($n > 6$, three independent experiments; $p = 0.004$ for CD4⁺CD25⁺ and $p = 0.029$ for CD4⁺Foxp3⁺ cell reduction). **(B)** Impaired upregulation of CD25a and Foxp3 upon in vitro stimulation of CD18^{hypo} PL/J total thymocytes with IL-2 and IL-2 + anti-CD3 for 24 h by FACS analysis (gated on total live thymocytes; $n = 4$, two independent experiments). CD18^{wt}, dashed gray line; CD18^{hypo}, straight black line. Bar graphs show significantly reduced frequencies of CD4⁺CD25^{high+} and CD4⁺Foxp3⁺ CD18^{hypo} PL/J thymocytes after stimulation with IL-2 and anti-CD3 compared with CD18^{wt} PL/J cell frequencies, indicating a potential defect of CD4⁺CD25^{high+} nTreg precursor generation in CD18^{hypo} PL/J thymi. **(C)** Reduced Stat5 phosphorylation (pY694) in CD18^{hypo} PL/J CD4⁺ thymocytes upon in vitro stimulation with IL-2 for 30 min in FACS analyses ($n = 4$, two independent experiments; $p = 0.001$). ** $p < 0.05$, *** $p < 0.01$. unstim, Unstimulated.

against Foxp3 and IL-17. Although CD18^{wt} PL/J skin harbored only a few isolated Foxp3⁺ and almost no IL-17⁺ cells, cell numbers of Foxp3⁺ and IL-17⁺ cells were increased in CD18^{hypo} PL/J skin, with some Foxp3⁺IL-17⁺ DP cells being present (Fig.

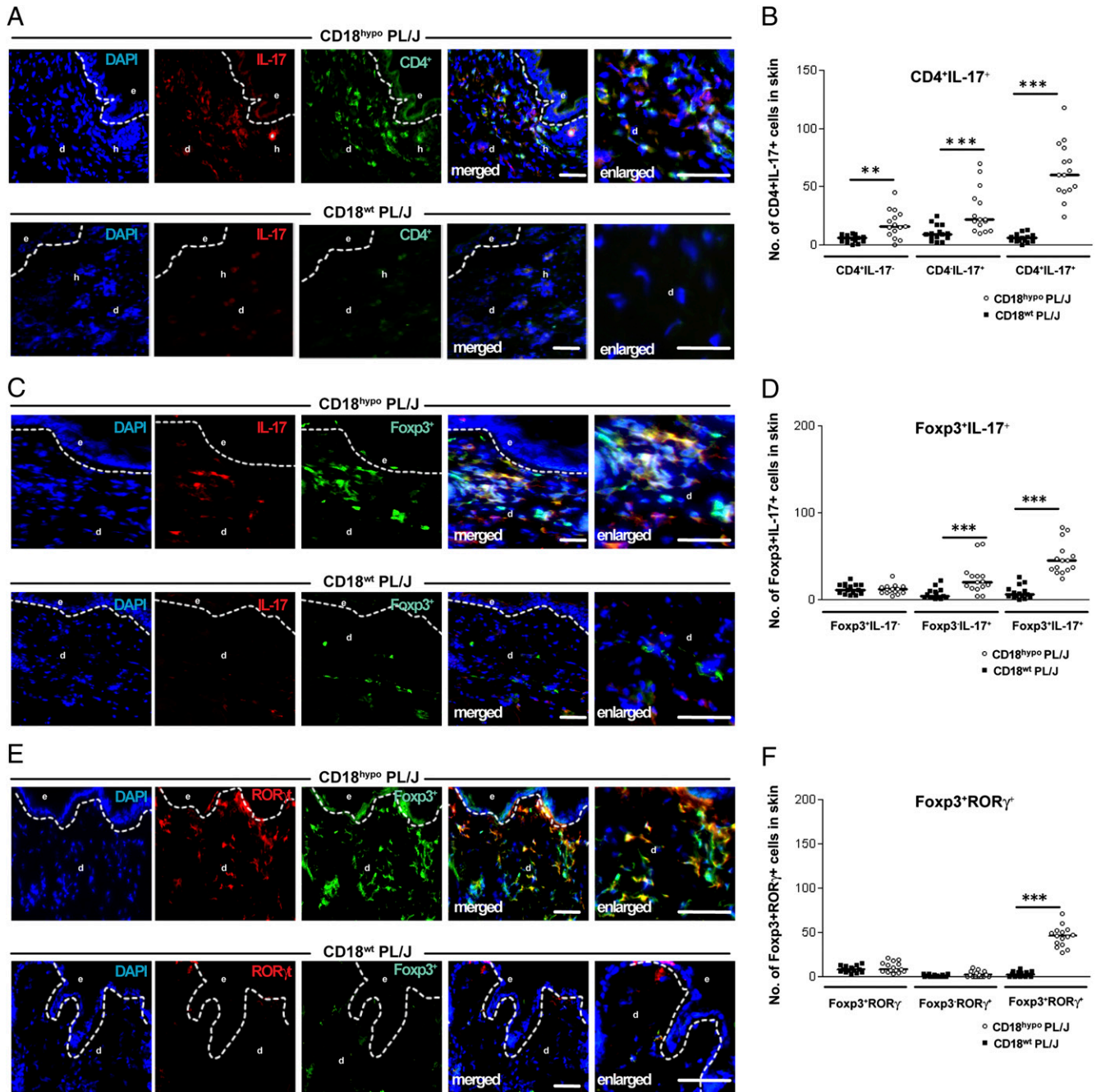


FIGURE 2. Infiltration of lesional skin of CD18^{hypo} PL/J mice with IL-17-producing CD4⁺ T cells and conversion of Foxp3⁺ Tregs into IL-17⁺ cells in situ. Representative microphotographs are depicted from skin cryosections. These are representative for three independent experiments with skin sections from at least three individual mice of each genotype. The white dotted lines indicate the borders between epidermis (e), dermis (d), and hair follicles (h). **(A)** Remarkably increased numbers of CD4⁺IL-17⁺ T cells are observed in lesional skin of CD18^{hypo} PL/J mice compared with healthy skin of CD18^{wt}PL/J mice by immunostaining (CD4⁺ T cells, green; IL-17⁺ cells, red; DAPI-stained nuclei, blue; original magnification $\times 40$). **(B)** To quantify CD4⁺IL-17⁺, CD4⁺IL-17⁻, and CD4⁺IL-17⁺ cells in the skin of CD18^{hypo} and CD18^{wt} PL/J mice, positively stained cells were counted. For all measurements, the median of specifically stained T cells counted in 15 high-power fields is presented ($n \geq 3$). **(C)** Infiltration of CD18^{hypo} PL/J skin with Foxp3⁺ and IL-17⁺ T cells. Presence of Foxp3⁺IL-17⁺ DP cells indicates the conversion of Foxp3⁺ Tregs into Th17 cells in inflamed skin (Foxp3⁺ cells, green; IL-17⁺ cells, red; DAPI-stained nuclei, blue; Foxp3⁺IL-17⁺ cells, yellow; original magnification $\times 40$). **(D)** To quantify Foxp3⁺IL-17⁻, Foxp3⁺IL-17⁺, and Foxp3⁺IL-17⁺ cells in the skin of CD18^{hypo} and CD18^{wt} PL/J mice, positively stained cells were counted. For all measurements, the median of specifically stained T cells counted in 15 high-power fields is presented ($n \geq 3$). **(E)** Significantly higher numbers of Foxp3⁺RORγ^t DP cells are present in inflamed skin of CD18^{hypo} compared with healthy skin of CD18^{wt}PL/J mice as detected by immunostaining (Foxp3⁺ cells, green; RORγ^t cells, red; DAPI-stained nuclei, blue; original magnification $\times 40$). **(F)** For quantification of Foxp3⁺RORγ^t, Foxp3⁺RORγ⁻, and Foxp3⁺RORγ^t cells, positively stained cells were counted. For all measurements, the median of specifically stained cells counted in 15 high-power fields is presented ($n \geq 3$). Scale bars, 100 μ m; 300 μ m in the corresponding enlarged microphotographs. ** $p < 0.05$, *** $p < 0.01$.

2C, 2D), indicating that Treg/Th17 conversion takes either place in vivo in affected skin or that converting Tregs migrate to the skin of CD18^{hypo} PL/J mice at increased rates. To confirm the lineage

duality of Foxp3⁺ IL-17-producing cells detected in affected CD18^{hypo} PL/J skin and rule out unspecific absorption of IL-17 on the surface of activated CD4⁺ cells that in some instances mas-

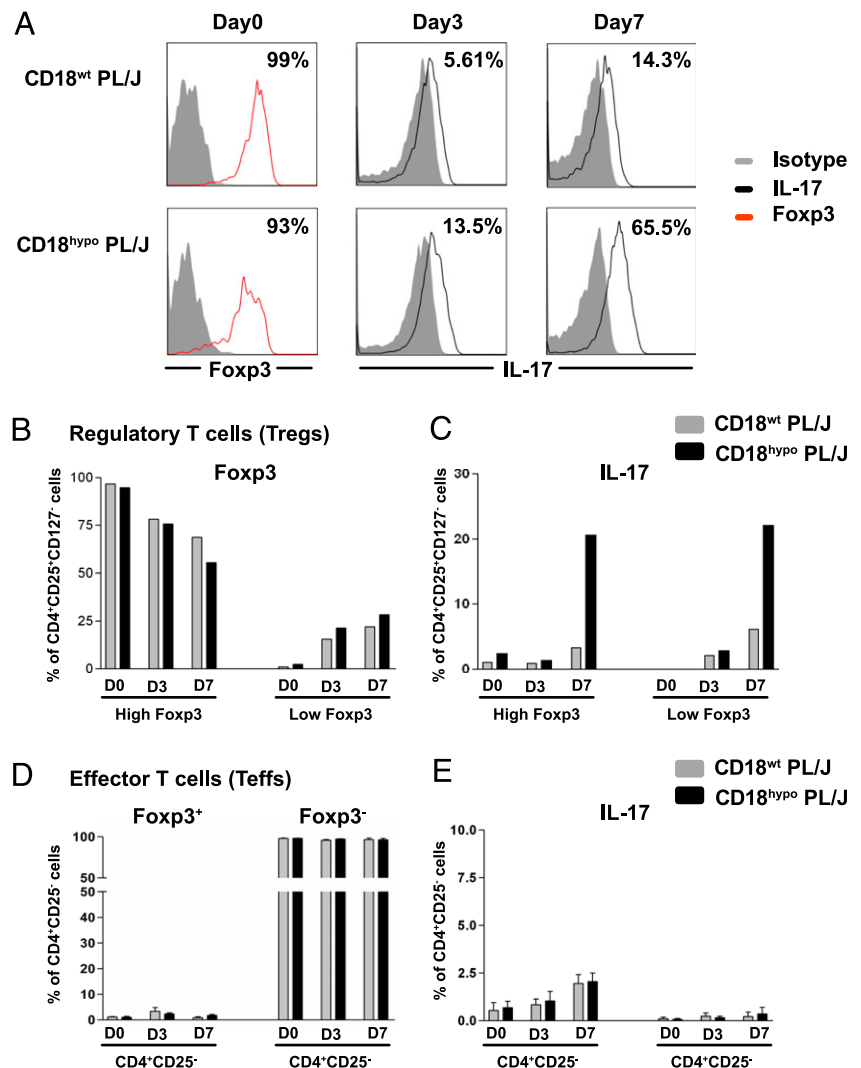
querade as Th17 cells (26), we subsequently also examined co-expression of ROR γ t, the key TF required for differentiation of Th17 cells, with Foxp3 in the skin infiltrate (27). Costaining of skin cryosections with Abs against Foxp3 and ROR γ t revealed a significantly higher presence of Foxp3⁺ROR γ t⁺ DP cells in inflamed CD18^{hypo} compared with CD18^{wt} PL/J skin that either differentiated from Foxp3⁺ Tregs in psoriasisform skin (Fig. 2E, 2F) or immigrated from skin-draining lymph nodes also harboring increased fractions of Foxp3⁺ROR γ t⁺ DP cells (Supplemental Fig. 1).

Reduced CD18 levels increase the propensity of CD18^{hypo} Tregs to lose Foxp3 and differentiate into proinflammatory IL-17⁺ cells in in vitro Treg–DC stimulation assays

For the in vitro analyses of Treg differentiation, a highly pure and well-defined population of murine CD4⁺CD25⁺CD127[−] Tregs was freshly isolated from spleens of CD18^{hypo} or CD18^{wt} PL/J mice using the methods described above and subsequently cocultured with allogeneic bone marrow–derived irradiated DCs from BALB/c mice in the presence of 500 U/ml recombinant murine IL-2. IL-2 is required for Treg maintenance and expansion, but is also capable of inducing Th17 conversion (28). Foxp3 and IL-17A expression were analyzed on days 0, 3, and 7 by intracellular cytokine staining and subsequent FACS analysis. The purity of the Treg starting population used for the Treg/Th17 differentiation experiments was generally >93% on day 0 as confirmed by their CD4⁺CD25⁺CD127[−]Foxp3⁺ phenotype by FACS analysis (Fig.

3A, left panel). By trend, CD18^{hypo} PL/J Tregs expressed slightly higher IL-17 levels on day 0, possibly resulting from an endogenous defect or from the inflammatory milieu in diseased mice. After the 7-d stimulation period, the resulting total CD4⁺ population of the CD18^{hypo} PL/J samples contained a higher total fraction of IL-17⁺ cells compared with CD18^{wt} PL/J samples (Fig. 3A, right panel) corresponding to a differentiation process of the original Foxp3^{high+} Tregs already detectable to some extent on day 3 (Fig. 3A, middle panel). By day 3 of the stimulation, the initial homogenous CD4⁺CD25⁺CD127[−]Foxp3^{high+} population moreover consistently started differentiating into a CD4⁺CD25⁺CD127[−]Foxp3^{high} and a -Foxp3^{low} population in all three individual experiments performed (Fig. 3B). Among these two populations, both Foxp3^{high} and Foxp3^{low} cells expressed IL-17A (Fig. 3C). However, on day 7 following allogeneic stimulation, CD4⁺CD25⁺CD127[−]Foxp3^{low} T cells derived from CD18^{hypo} PL/J mice were capable of enhanced production of IL-17 compared with CD4⁺CD25⁺Foxp3^{high} T cells. On average, an increase of IL-17–expressing cells by 10–20% and a decrease of Foxp3 expression by 50–70% in CD18^{hypo} PL/J Tregs were detected compared with CD18^{wt} PL/J Tregs. Importantly, dividing Tregs expressed IL-17 and gradually lost Foxp3 expression, supporting the concept that SP IL-17⁺ cells underwent a Foxp3⁺IL-17⁺ DP stage (8). However, by day 7, an inverse relationship of Foxp3- and IL-17–expressing cells could be observed in Treg–DC coculture compared with day 0.

FIGURE 3. Increased propensity of CD18^{hypo} PL/J CD4⁺CD25⁺ Tregs to lose Foxp3 and increase IL-17A expression in in vitro Treg–DC coculture assays. (A)–(C) show representative experiments for differentiation of Tregs into IL-17–producing Th17 cells. Similar results were reproducible in three independent experiments. Light gray and black bars represent CD18^{wt} PL/J and CD18^{hypo} PL/J CD4⁺CD25⁺ T cells, respectively. (A) The purity of CD4⁺CD25⁺CD127[−]Foxp3⁺ Tregs was >93% for all CD18^{hypo} and CD18^{wt} samples as indicated by their Foxp3⁺ status (left panel). On day 7 after allogeneic stimulation of either CD18^{hypo} PL/J or CD18^{wt} PL/J Tregs with BALB/c DC in MLRs in the presence of IL-2, the resulting CD18^{hypo} PL/J CD4⁺ cell population had higher expression levels of IL-17 than CD4⁺ CD18^{wt} PL/J cells (black lines) compared with isotype IgG controls (gray; right panel). The difference in IL-17 expression between CD18^{wt} and CD18^{hypo} PL/J Tregs was already detectable on day 3 (middle panel). Histograms represent IL-17 levels of the total CD4⁺ cell population for both genotypes. (B) The homogeneous Treg population at day 0 consistently divided into a CD4⁺CD25⁺Foxp3^{high} and CD4⁺CD25⁺Foxp3^{low} population during 7 d of stimulation with IL-2 in the presence of BALB/c DC. (C) Tregs derived from CD18^{hypo} PL/J possessed an enhanced tendency to lose Foxp3 and differentiate into IL-17–producing Th17 cells. Notably, Foxp3^{low} cells expressed higher IL-17A levels compared with Foxp3^{high} cells. (D) IL-2 stimulation of CD4⁺CD25[−] Teffs from either mouse strain for 7 d in MLR did not result in any significant induction CD4⁺Foxp3⁺ Tregs. (E) Similarly, only marginal differentiation of CD4⁺CD25[−] Teffs from either CD18^{hypo} PL/J or CD18^{wt} PL/J spleens into Th17 cells was observed in MLRs in the presence of IL-2 (please note the different scale).



In parallel experiments, we also analyzed CD4⁺CD25[−] splenic T cells (effector T cells [Teffs]) for their ability to differentiate into IL-17–producing cells. To this end, CD4⁺CD25[−] T cells from spleens of either CD18^{hypo} or CD18^{wt} PL/J mice were cocultured with allogeneic bone marrow–derived irradiated DCs from BALB/c mice in the presence of 500 U/ml recombinant murine IL-2 as described above. No significant increase of IL-17–producing cells was observed, and the IL-17–positive T cell fraction was below 1–1.5% on day 7 independent from the CD18 status (Fig. 3D, 3E). Instead of an inverse regulation of Foxp3 and IL-17 as detected in CD4⁺CD25⁺ Tregs, a slight, but not significant, increase in Foxp3 expression of CD4⁺CD25[−] Teffs was observed over time. In summary, a comparable inverse regulation of Foxp3 and IL-17 or induction of IL-17A as detected in CD4⁺CD25⁺ Tregs could not be established for the CD4⁺CD25[−] Teff population.

We further analyzed whether reduced expression of CD18 on DCs derived from CD18^{hypo} mice promotes Treg differentiation into IL-17–producing Th17 cells and might contribute to the Treg conversion in vivo. For this purpose, Tregs derived from BALB/c mice were cocultured with allogeneic DCs obtained from CD18^{hypo} PL/J and CD18^{wt} PL/J mice as described above. Low basal IL-17 production could be detected in Tregs derived from BALB/c mice on day 0, but no major increase in IL-17 production occurred by day 7 independent from the CD18 levels on PL/J DCs (Supplemental Fig. 2). Instead, an increase of the Foxp3⁺–population by 20–30% was observed without noticeable inversion of the ratio of Foxp3⁺ to IL-17–producing cells. In follow-up experiments, we analyzed CD18^{hypo} and CD18^{wt} PL/J Tregs in coculture with allogeneic CD18^{hypo} and CD18^{wt} C57BL/6J DCs and again could not detect any significant induction of Treg conversion by CD18^{hypo} DCs (Supplemental Fig. 3).

Blocking of CD18 with neutralizing anti-CD18 Ab induces conversion of CD18^{wt} Tregs into IL-17A–producing cells in Treg–DC cocultures in vitro

To confirm that the enhanced differentiation of CD18^{hypo} PL/J Tregs into Th17 cells observed in vitro is a consequence of reduced CD18 expression (i.e., CD18-dependent) and to analyze the dose-response effect of diminished CD18 levels on Treg plasticity, we used anti-CD18 blocking Abs in cocultures of allogeneic DCs and Tregs derived from CD18^{wt} PL/J mice and, for comparison, also from CD18^{hypo} PL/J mice. Increasing concentrations of CD18 blocking Abs (0, 5, 10, and 20 μ g/ml) were added to Treg–DC cocultures in the presence of 500 U/ml recombinant murine IL-2. In a control experiment, isotype control Abs at identical concentrations were used instead of anti-CD18 Abs. Expression of IL-17 was analyzed on days 0, 3, and 7 via FACS as described above. Notably, a significant upregulation of IL-17 in CD18^{wt} PL/J Tregs was dose-dependently observed on day 7 upon addition of neutralizing anti-CD18 Abs (Fig. 4A) with concomitant decrease of Foxp3 levels (Fig. 4B, representative experiments are shown). Isotype control IgG did not induce any significant conversion of CD18^{wt} PL/J Tregs into Foxp3^{low} IL-17⁺ T cells. Moreover, anti-CD18 Abs did not cause in any significant increase in Treg apoptosis, and apoptotic cell fractions were generally <10% upon addition of all concentrations of anti-CD18 used in this study (0–20 μ g/ml) (data not shown). These results confirm a direct influence of the reduced CD18 expression on differentiation of Tregs into Th17 cells in the CD18^{hypo} PL/J mouse model. In a follow-up experiment, splenic Tregs from three individual CD18^{wt} PL/J mice were differentiated into IL-17⁺ cells applying 20 μ g/ml anti-CD18 in the presence of IL-2 to statistically assess the reproducibility of the conversion effect. Tregs from all three mice had converted into IL-17⁺Foxp3^{low} cells to similar extents on day 7 following anti-

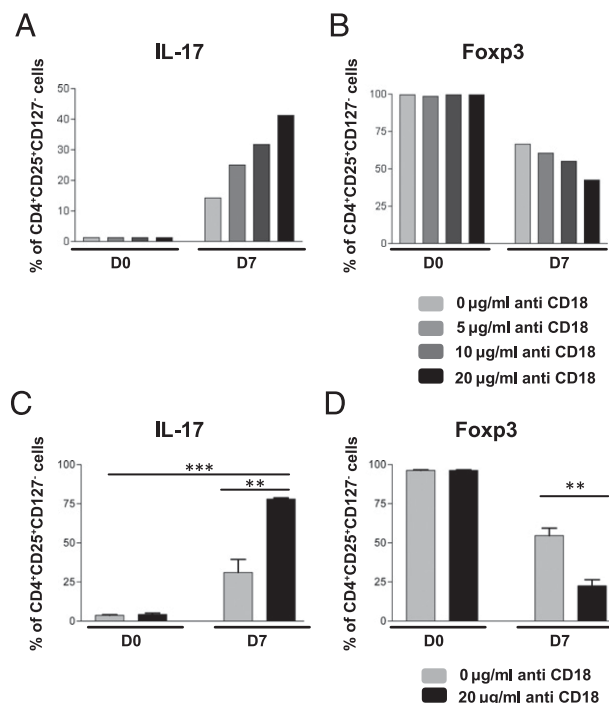


FIGURE 4. Conversion of CD18^{wt} PL/J Tregs into IL-17A–producing cells is inducible upon blocking CD18 with neutralizing Abs in vitro Treg–DC coculture. **(A)** Increasing concentrations of neutralizing Abs against CD18-induced conversion of CD18^{wt} PL/J Tregs into IL-17A–producing cells in a dose-dependent manner in cocultures of CD18^{wt} PL/J Tregs and BALB/c DC in the presence of IL-2 (c[anti-CD18] = 0, 5, 10, and 20 μ g/ml). These representative data were reproducible in three independent experiments. **(B)** Dose-dependent loss of Foxp3 expression of CD18^{wt} PL/J Tregs in response to incubation with indicated doses of anti-CD18 for 7 d in MLRs in the presence of IL-2. **(C and D)** Reproducibility of Th17 conversion upon stimulation of CD18^{wt} PL/J Tregs with neutralizing anti-CD18 Abs in MLRs. IL-17 and Foxp3 expression of Tregs derived from three individual CD18^{wt} PL/J spleens after exposure to 20 μ g/ml anti-CD18 Abs for 7 d was compared. Upregulation of IL-17 and downregulation of Foxp3 occurred consistently in up to 80% of Tregs. ** p < 0.05, *** p < 0.01.

CD18 exposure with statistically highly significant increases in IL-17⁺ fractions and corresponding decreases in Foxp3⁺ fractions by up to ~80% (Fig. 4C, 4D).

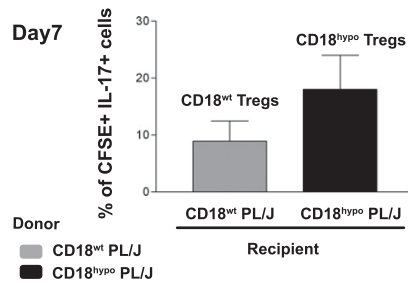
Adoptively transferred CFSE-labeled Tregs derived from CD18^{hypo} PL/J mice differentiate into IL-17A–producing Th17 cells in vivo

To track the differentiation of Tregs into IL-17A–producing Th17 cells in vivo, Tregs purified from spleens of CD18^{wt} PL/J or CD18^{hypo} PL/J mice were labeled with CFSE as described above. For the homologous transfer experiments, a total of 1×10^6 CFSE-labeled Tregs from CD18^{wt} and CD18^{hypo} PL/J mice were injected i.v. into CD18^{wt} PL/J and CD18^{hypo} PL/J recipients, respectively. On day 7, recipient mice were analyzed for IL-17 production in CFSE-labeled cells recovered from spleens. Consistent with our in vitro results, we observed an enhanced production of IL-17 in CD18^{hypo} Tregs injected into CD18^{hypo} PL/J mice in comparison with CD18^{wt} Tregs in CD18^{wt} PL/J mice (Fig. 5A). Notably, cells with lower CFSE fluorescence intensity on day 7 produced higher levels of IL-17, indicating that proliferating cells had preferentially differentiated into IL-17–producing cells.

To investigate a potential influence of the inflammatory environment on the differentiation process of Tregs, in a follow-up experiment, CFSE-labeled Tregs derived from CD18^{wt} and

A

Homologous adoptive transfer of CFSE labeled Tregs



B

Reciprocal adoptive transfer of CFSE labeled Tregs

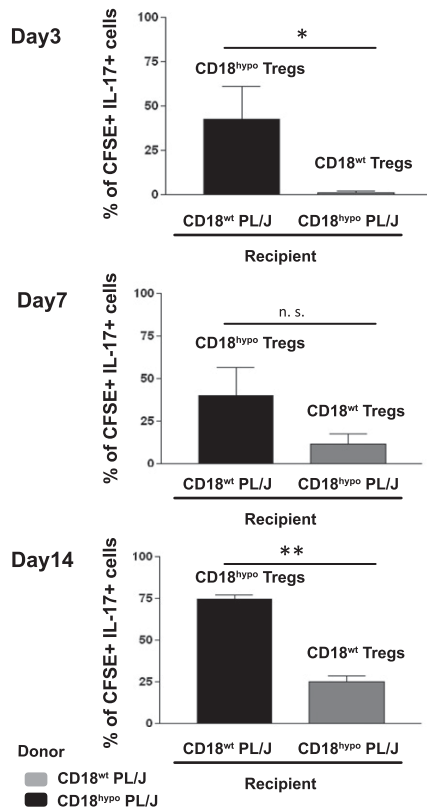


FIGURE 5. Adoptively transferred CFSE-labeled Tregs derived from CD18^{hypo} PL/J mice displayed enhanced conversion into IL-17A-producing Th17 cells in vivo compared with CD18^{wt} PL/J Tregs. 1×10^6 CFSE labeled Tregs of either mouse strain were injected into CD18^{wt} or CD18^{hypo} PL/J recipients, respectively. On day 7 after transfer or as indicated, recipient mice were analyzed for IL-17 production in CFSE labeled Tregs ($n \geq 3$ per mouse line and per time point). **(A)** Homologous transfer of Tregs. Enhanced production of IL-17 in CD18^{hypo} Tregs injected into CD18^{hypo} PL/J mice compared with CD18^{wt} Tregs in CD18^{wt} PL/J mice. **(B)** Reciprocal transfer of Tregs. CFSE-labeled Tregs derived from either CD18^{wt} or CD18^{hypo} PL/J mice were injected reciprocally into CD18^{hypo} or CD18^{wt} PL/J recipients. Increased percentages of IL-17-producing CFSE-labeled CD18^{hypo} T cells were detectable in CD18^{wt} PL/J recipients already on day 3 after transfer, confirming that Tregs derived from CD18^{hypo} PL/J mice display an enhanced propensity to differentiate into IL-17-producing cells compared with CD18^{wt} PL/J Tregs, even in a noninflammatory environment. By day 14 posttransfer, conversion of CFSE-labeled CD18^{hypo} Tregs to IL-17-producing T cells had further increased in CD18^{wt} PL/J hosts compared with CD18^{wt} Tregs in CD18^{hypo} PL/J hosts as indicated by the higher percentages of CD18^{hypo} CFSE+IL-17+ cells. This experiment was performed on three individual mice of each genotype. * $p < 0.1$, ** $p < 0.05$.

CD18^{hypo} PL/J mice were injected reciprocally into either CD18^{hypo} PL/J or CD18^{wt} PL/J recipients, respectively. Increased percentages of IL-17-producing CFSE-labeled CD18^{hypo} Tregs were found in CD18^{wt} PL/J recipients on days 3, 7, and 14 posttransfer, confirming that Tregs derived from CD18^{hypo} PL/J mice display an enhanced intrinsic propensity to differentiate into IL-17-producing Th17 cells compared with CD18^{wt} Tregs (Fig. 5B). On day 14 post-reciprocal transfer, the frequency of IL-17-producing CFSE-labeled CD18^{wt} as well as CD18^{hypo} Tregs had further increased compared with days 3 and 7, confirming that Treg conversion was maintained over time. The significantly greater increase in conversion rates of CD18^{hypo} Tregs by day 14 compared with CD18^{wt} Tregs verified that the differences in Foxp3⁺IL-17⁺ converters between CD18^{hypo} and CD18^{wt} PL/J mice were not attenuated with time, but actually became even more profound, further supporting our concept of promotion of Treg/Th17 conversion by suboptimal CD18 levels. Interestingly, we did not observe any major influence of the proinflammatory environment on CD18^{hypo} PL/J Treg plasticity, but instead, Treg differentiation into Th17 cells seemed to be primarily dependent on CD18 expression levels in our model.

Discussion

Previous investigations using CD18^{-/-} mice established critical roles for CD18/β2 integrins in the development of optimal numbers of thymic and peripheral CD4⁺CD25⁺ T cells as well as for optimal function of murine CD4⁺CD25⁺ Tregs in vitro and in vivo (29). Complete absence of CD18 on CD4⁺CD25⁺ T cells was associated with defects in immune homeostasis and the subsequent development of autoimmune diseases, in particular autoimmune colitis and ileitis (29). We demonstrate in this study that reduced expression of CD18 to 2–16% of wild-type levels similarly impacts the development of nTregs and, most interestingly, facilitates differentiation of peripheral Treg into IL-17-producing Th17 cells. Using the CD18^{hypo} PL/J murine psoriasis model, we found a dose-dependent relationship between CD18 expression levels on Tregs and their propensity to convert into IL-17-producing cells. The dose effect of CD18 expression on differentiation of Tregs into IL-17-producing cells was further substantiated in MLR in vitro when Tregs derived from CD18 competent mice in the presence of neutralizing Abs against CD18 dose-dependently adopted a Th17 phenotype. To our knowledge, this is the first study showing an impact of CD18 levels on Treg/Th17 plasticity and a pathogenic consequence in the murine CD18^{hypo} PL/J model of psoriasis.

CD4⁺CD25^{high+}Foxp3⁺ Tregs critically contribute to the maintenance of immune homeostasis and immunological self-tolerance through suppression of pathological and physiological immune response. Thymic-derived nTregs and iTregs work in collaboration to prevent autoimmune responses (30). The role of Foxp3 as key regulator of development and function of nTregs is quite evident, but its role in the differentiation of iTregs is less clear. In the present investigation, we further explored the relationship between Foxp3 expression levels and Th17 differentiation of Tregs in the CD18^{hypo} PL/J mouse model and successfully established a role for CD18 in Treg plasticity. Our results thus provide additional insights into pathogenic mechanisms of psoriasiform skin disease in the CD18^{hypo} PL/J mouse model, which most likely will be relevant to understanding human psoriasis. In fact, reduced CD18 expression levels have been identified on leukocytes in psoriasis patients (31–33), and in an independent study, the conversion of Tregs into Th17 cells was convincingly shown in psoriasis patients (24). In contrast to these rather correlative data, we provide in this study direct evidence for a causal relationship between CD18

expression levels and the enhanced conversion of Tregs into Th17 cells enhancing the psoriasiform phenotype in CD18^{hypo} PL/J mice. Although a complete block of CD11a/CD18 (LFA-1), previously also used therapeutically by anti-psoriatic drugs such as the Ab efalizumab (34), primarily affects migration of autoreactive lymphocytes into the skin and may lead to symptoms of immunodeficiency comparable to human leukocyte adhesion deficiency as a consequence of decreased T cell activation, suboptimal CD18 function, and impaired interaction with its ICAM ligands on DCs—that is, mimicked in the CD18^{hypo} PL/J mouse model and potentially also occurs in psoriasis patients as a consequence of altered ICAM and/or CD18 expression (17, 35)—results in the Treg dysfunction described in this study and in autoimmunity such as psoriasiform skin disease in mice. Notably, apart from sporadic cases of progressive multifocal leukoencephalopathy, severe relapses of pustular psoriasis were reported in patients treated with efalizumab (34), possibly indicating that under nonsaturating efalizumab conditions, activated Tregs rush into the skin, whereas Tregs may be still suppressed or may not have functionally recovered from efalizumab treatment yet. In fact, due to these side effects, efalizumab has been withdrawn from the market.

The healthy thymus is capable of producing functional nTregs, but may also give rise to autoreactive, potentially pathogenic T cells. Although DP thymocytes with a high affinity to self-ligands presented by thymic epithelial cells are normally deleted through negative selection, thymocytes with an intermediate affinity for self-Ags that receive sufficient stimulation by TGF- β and IL-2 may become nTregs (recently reviewed in Ref. 36). This selection process can be influenced by negative regulatory signals modifying the TCR signaling threshold in thymocytes (37). Mature Tregs persist in the periphery and exert dominant control over autoreactive T cells. Treg deficiency in the periphery is sufficient to evoke chronic T cell–mediated autoimmunity and immune pathology (8). Our findings that reduced expression of CD18 in the CD18^{hypo} PL/J mouse model results in reduced numbers of nTreg precursors and CD4⁺Foxp3⁺ nTregs in thymi of young CD18^{hypo} PL/J prior to the manifestation of psoriasiform dermatitis confirm a role for CD18 in early Treg commitment, likely through regulation of cell–cell contacts between Treg precursors and thymic stromal cells, modification of TCR signals, or through paracrine regulation of the abundance of cytokine-like IL-2 or IL-7 signals at the immunological synapses between developing Tregs and thymic stromal cells. Because LFA-1 enhances the affinity/avidity of the TCR for its MHC class II–peptide ligand by stabilizing T cell–DC contacts (38), a reduction of CD18 in the CD18^{hypo} PL/J model might shift the threshold of selection in DP thymocytes, with fewer CD4⁺CD25⁺ Treg precursors being selected and subsequent impact on Foxp3 upregulation.

IL-2R signaling is essential for the development, homeostasis, and activation of nTregs (39, 40). IL-2 maintains Foxp3⁺ nTregs and facilitates TGF- β –dependent differentiation of naive T cells to Foxp3⁺ iTregs. Binding of IL-2 to its receptor induces phosphorylation of the TF Stat5, and p-Stat5 (pY694/pY699) activates Foxp3 transcription at the Foxp3 promoter, subsequently resulting in elevated levels of Foxp3 protein in Tregs (8, 41, 42). Our results indicate a contribution of CD18 to Stat5-dependent nTreg generation in the thymus via impaired upregulation of CD25a/IL-2R α . Diminished CD25a/IL-2R α upregulation in CD18^{hypo} PL/J thymocytes could be confirmed in *in vitro* stimulation assays with anti-CD3 and IL-2. Interestingly, generation of CD4⁺CD25^{high}⁺ nTreg precursors from CD18^{hypo} PL/J thymocytes is possible, but the total ratio of CD4⁺CD25⁺ cells is reduced. The exact molecular basis of defective CD25a/IL-2R α upregulation in CD18^{hypo}

PL/J T cells still needs to be analyzed in more detail and might involve alterations in calcium flux, calcineurin, and NF-AT signaling due to the suboptimal TCR stimulation in the context with an avidity/affinity shift of the precursor population.

Ziegler and Buckner (43) provided evidence that peripheral CD4⁺ T cell differentiation into Treg or Th17 cells depends on the environment in which activation occurs. The balance between Foxp3 and ROR γ t levels finally determines CD4⁺ T cell fate and the type of immune response that will be generated. Foxp3 is capable of physically associating with ROR γ t and inhibiting the ability of ROR γ t to act as a transcriptional activator (43, 44), thereby establishing an inverse relationship between Foxp3 level and Th17 differentiation. We consistently found that Foxp3 levels were decreased in CD18^{hypo} PL/J Tregs, most likely resulting in an imbalance between Foxp3 and ROR γ t, facilitating conversion of peripheral Tregs into Th17 cells. Furthermore, the increased presence of Foxp3⁺ROR γ t⁺ DP cells in skin and lymph nodes of affected CD18^{hypo} PL/J mice detected in our *in situ* immunohistochemical analyses provides direct evidence of lineage duality and impressively illustrates the peripheral conversion process of Tregs in this psoriasis mouse model. Although Ag presentation to Tregs and interaction with DCs might take place in the skin-draining lymph nodes of CD18^{hypo} PL/J mice and induce the Treg/Th17 conversion process, we observed increased numbers of Foxp3⁺ SP as well as of Foxp3⁺IL-17⁺ and Foxp3⁺ROR γ t⁺ DP cells in inflamed CD18^{hypo} PL/J skin, suggesting that, at least in part, Treg conversion actually occurs in the skin itself. Alternatively, Treg conversion might take place in secondary lymphoid organs other than the skin, in particular in lymph nodes, and converting cells migrate into CD18^{hypo} PL/J skin at enhanced rates due to alterations in trafficking and expression of skin-homing molecules in this psoriasis mouse model. As a consequence of the CD18^{hypo} mutation, trafficking of immune cells into the skin could be impaired, but levels of other surface receptors and chemokines involved in skin homing such as CCR6 or cutaneous lymphocyte Ag and CCL20 were found at increased levels in CD18^{hypo} PL/J skin (M. Gatzka, unpublished observations). In addition to the increased intrinsic propensity of CD18^{hypo} Tregs to convert into Th17 cells confirmed in our *in vitro* experiments, altered migration and expression of chemokine receptors on Tregs *in vivo* therefore likely contribute to the skin inflammation developing in CD18^{hypo} PL/J mice (45–47).

In the presence of IL-6, inhibition of ROR γ t by Foxp3 is abrogated, leading to initiation of Th17 differentiation (41). The pivotal role of IL-6 in determining the balance between Tregs and Th17 cells could also be confirmed by another group in a model of experimental autoimmune encephalomyelitis (48). A comparable process might trigger the onset of psoriasiform skin disease in CD18^{hypo} PL/J mice by favoring Foxp3⁺ Treg conversion into Th17 cells, gradually leading to a preponderance of inflammatory signals. In fact, increased levels of proinflammatory cytokines such as IL-6 and IL-23 in peripheral lymphoid organs of CD18^{hypo} compared with CD18^{wt} PL/J mice create an inflammatory environment (Supplemental Fig. 1 and data not shown). It is interesting though, that even in CD18^{wt} PL/J mice, CD18^{hypo} PL/J Tregs adoptively transferred converted into IL-17–producing cells at greater rates than CD18^{wt} PL/J Tregs independent from inflammatory signals. Nonetheless, high levels of inflammatory cytokines in the skin might further promote inhibition of Foxp3 function in CD18^{hypo} PL/J Tregs and collaborate with the intrinsic ability of Tregs with low CD18 expression levels, thereby inducing the Th17 differentiation pathway.

Our previous results indicate that reduced CD18 expression levels might impair peripheral cell–cell interactions between DCs

and Tregs as well as interactions between Tregs and Teffs, leading to reduced Treg function and onset of psoriasiform dermatitis in CD18^{hypo} PL/J mice (6). Further exploration of the effect of reduced CD18 expression on DCs and their role in Th17 differentiation using CD18^{hypo} PL/J DCs and BALB/c Tregs did not reveal any crucial involvement of CD18 on DC in Treg conversion into Th17 cells, but rather suggests an intrinsic role for CD18 in Tregs. Low CD18 expression on Tregs from CD18^{hypo} PL/J mice may impair TCR activation, eventually leading to reduced proliferation, activation, and suppressor function of Tregs (6); however, the exact molecular pathways are still unknown and need to be examined in greater detail. In a previous investigation assessing the suppressor function of CD18^{hypo} PL/J Tregs, we in particular also found a reduced ability to produce TGF- β 1 (6), a cytokine known to influence the Treg/Th17 balance (49, 50).

To identify potential peripheral mechanisms for the observed decline of Treg numbers in affected CD18^{hypo} PL/J mice, we then further analyzed the impact of reduced CD18 expression on differentiation of a defined population of peripheral Tregs (CD4⁺CD25⁺CD127⁻Foxp3⁺) into Th17 cells. Interestingly, adding CD18 blocking Abs to CD18^{wt} PL/J Tregs in vitro caused a dose-dependent increase in IL-17A-producing cells in Treg–DC cocultures compared with isotype control Ab. These data indicate that reduced CD18 expression previously reported to impair the synapse formation between Treg and DCs (6) also promotes the differentiation of IL-17–producing Th17 cells. Thus, it is likely that impaired synapse formation between Tregs and DCs is responsible for the deviant Th17 differentiation. We therefore conclude that due to reduced CD18 levels, Foxp3 expression of Tregs gradually diminishes, and in an inflammatory environment, CD18^{hypo} PL/J Tregs eventually convert into IL-17–producing Th17 cells. These in vitro results were confirmed by our analyses of differentiation of Foxp3⁺ Tregs into Th17 cells in the CD18^{hypo} PL/J mouse model in vivo. Accordingly, adoptive transfer of CFSE-labeled CD18^{wt} and CD18^{hypo} Tregs into CD18^{wt} PL/J and CD18^{hypo} PL/J mice consistently revealed enhanced conversion of CFSE-labeled CD4⁺CD25⁺CD127⁻ Tregs derived from CD18^{hypo} PL/J mice into Th17 cells when compared with CD18^{wt} PL/J Tregs. However, in a reciprocal experiment, Tregs derived from CD18^{hypo} in CD18^{wt} PL/J displayed significant induction of IL-17 expression, which again confirms the higher tendency of CD18^{hypo} PL/J Tregs to convert into IL-17–producing cells in comparison with CD18^{wt} Tregs. Consequently, there is a good correlation between our in vitro MLR data (Figs. 3, 4, Supplemental Fig. 4) and the in situ (Fig. 2) and in vivo data (Fig. 5), overall convincingly showing that CD18^{hypo} Tregs have an increased tendency to convert into Th17/IL-17–producing cells.

In summary, several CD18-dependent mechanisms that may jointly cause loss of Treg numbers and predominance of IL-17–producing T cells in CD18^{hypo} PL/J mice, thereby contributing to psoriasiform skin disease have been identified so far: 1) decreased production of nTregs already in young mice prior to the manifestation of psoriasiform dermatitis; 2) further reduction of thymic output of nTregs with age; 3) impaired peripheral Treg function and reduced production of TGF- β 1; and 4) conversion of Tregs into IL-17–producing Th17 cells in inflamed skin and peripheral lymphatic organs. The function of CD18^{hypo} PL/J DCs seemed to be normal in terms of their effect on Treg maintenance and induction of Treg proliferation. Impaired interaction of Treg and DCs results in improper Treg activation followed by differentiation of Tregs into Th17 cells and subsequent hyperactivation of Teffs (15, 16). These activated pathogenic Teffs, in the absence of fully functional Tregs, trigger a cascade of inflammatory events, including recruitment and activation of macrophages, with resulting

overproduction of the proinflammatory cytokine TNF- α , eventually leading to unrestrained amplification of inflammatory circuits and the manifestation of the psoriasiform skin disease (18). In conclusion, our data strongly confirm the central role of CD18 in T cell development, T cell activation, and regulation of autoimmunity. Additional work will now be performed to confirm the role of CD18 in Treg plasticity in human psoriasis patients as well as to directly track Treg conversion into Th17 cells in the skin of affected CD18^{hypo} PL/J mice using in vivo imaging techniques.

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Disclosures

The authors have no financial conflicts of interest.

References

- Schön, M. P., and W. H. Boehncke. 2005. Psoriasis. *N. Engl. J. Med.* 352: 1899–1912.
- Nickoloff, B. J. 1999. The immunologic and genetic basis of psoriasis. *Arch. Dermatol.* 135: 1104–1110.
- Nickoloff, B. J., and F. O. Nestle. 2004. Recent insights into the immunopathogenesis of psoriasis provide new therapeutic opportunities. *J. Clin. Invest.* 113: 1664–1675.
- Schön, M. P., M. Detmar, and C. M. Parker. 1997. Murine psoriasis-like disorder induced by naive CD4⁺ T cells. *Nat. Med.* 3: 183–188.
- Sugiyama, H., R. Gyulai, E. Toichi, E. Garaczi, S. Shimada, S. R. Stevens, Sr., T. S. McCormick, and K. D. Cooper. 2005. Dysfunctional blood and target tissue CD4⁺CD25⁺high regulatory T cells in psoriasis: mechanism underlying unstrained pathogenic effector T cell proliferation. *J. Immunol.* 174: 164–173.
- Wang, H., T. Peters, A. Sindrilu, D. Kess, T. Oreshkova, X. Z. Yu, A. M. Seier, H. Schreiber, M. Wlaschek, R. Blakytyn, et al. 2008. TGF- β -dependent suppressive function of Tregs requires wild-type levels of CD18 in a mouse model of psoriasis. *J. Clin. Invest.* 118: 2629–2639.
- Yun, W. J., D. W. Lee, S. E. Chang, G. S. Yoon, J. R. Huh, C. H. Won, M. W. Lee, S. E. Kim, B. J. Kim, K. C. Moon, and J. H. Choi. 2010. Role of CD4⁺CD25⁺FOXP3 regulatory T cells in psoriasis. *Ann. Dermatol.* 22: 397–403.
- Sakaguchi, S., T. Yamaguchi, T. Nomura, and M. Ono. 2008. Regulatory T cells and immune tolerance. *Cell* 133: 775–787.
- Josefowicz, S. Z., L. F. Lu, and A. Y. Rudensky. 2012. Regulatory T cells: mechanisms of differentiation and function. *Annu. Rev. Immunol.* 30: 531–564.
- Harrington, L. E., R. D. Hatton, P. R. Mangan, H. Turner, T. L. Murphy, K. M. Murphy, and C. T. Weaver. 2005. Interleukin 17–producing CD4⁺ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* 6: 1123–1132.
- Park, H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, Y. H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* 6: 1133–1141.
- Goodman, W. A., A. D. Levine, J. V. Massari, H. Sugiyama, T. S. McCormick, and K. D. Cooper. 2009. IL-6 signaling in psoriasis prevents immune suppression by regulatory T cells. *J. Immunol.* 183: 3170–3176.
- Rizzo, H. L., S. Kagami, K. G. Phillips, S. E. Kurtz, S. L. Jacques, and A. Blauvelt. 2011. IL-23-mediated psoriasis-like epidermal hyperplasia is dependent on IL-17A. *J. Immunol.* 186: 1495–1502.
- Korn, T., E. Bettelli, M. Oukka, and V. K. Kuchroo. 2009. IL-17 and Th17 Cells. *Annu. Rev. Immunol.* 27: 485–517.
- Wilson, R. W., C. M. Ballantyne, C. W. Smith, C. Montgomery, A. Bradley, W. E. O'Brien, and A. L. Beaudet. 1993. Gene targeting yields a CD18-mutant mouse for study of inflammation. *J. Immunol.* 151: 1571–1578.
- Bullard, D. C., K. Scharffetter-Kochanek, M. J. McArthur, J. G. Chosay, M. E. McBride, C. A. Montgomery, and A. L. Beaudet. 1996. A polygenic mouse model of psoriasiform skin disease in CD18-deficient mice. *Proc. Natl. Acad. Sci. USA* 93: 2116–2121.
- Kess, D., T. Peters, J. Zamek, C. Wickenhauser, S. Tawadros, K. Loser, G. Varga, S. Grabbe, R. Nischt, C. Sunderkötter, et al. 2003. CD4⁺ T cell-associated pathophysiology critically depends on CD18 gene dose effects in a murine model of psoriasis. *J. Immunol.* 171: 5697–5706.
- Wang, H., T. Peters, D. Kess, A. Sindrilu, T. Oreshkova, N. Van Rooijen, A. Stratis, A. C. Renkl, C. Sunderkötter, M. Wlaschek, et al. 2006. Activated

- macrophages are essential in a murine model for T cell-mediated chronic psoriasiform skin inflammation. *J. Clin. Invest.* 116: 2105–2114.
19. Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell* 48: 549–554.
 20. Carlos, T. M., and J. M. Harlan. 1994. Leukocyte-endothelial adhesion molecules. *Blood* 84: 2068–2101.
 21. Wang, H., D. Kess, A. K. Lindqvist, T. Peters, A. Sindrilaru, M. Wlaschek, R. Blakytyn, R. Holmdahl, and K. Scharffetter-Kochanek. 2008. A 9-centimorgan interval of chromosome 10 controls the T cell-dependent psoriasiform skin disease and arthritis in a murine psoriasis model. *J. Immunol.* 180: 5520–5529.
 22. van de Kerkhof, P. C., and C. M. Weemaes. 1990. Skin manifestations in congenital deficiency of leucocyte-adherence glycoproteins (CDLG). *Br. J. Dermatol.* 123: 395–401.
 23. Tomfohrde, J., A. Silverman, R. Barnes, M. A. Fernandez-Vina, M. Young, D. Lory, L. Morris, K. D. Wuepper, P. Stastny, A. Menter, et al. 1994. Gene for familial psoriasis susceptibility mapped to the distal end of human chromosome 17q. *Science* 264: 1141–1145.
 24. Bovenschen, H. J., P. C. van de Kerkhof, P. E. van Erp, R. Woestenrenk, I. Joosten, and H. J. P. M. Koenen. 2011. Foxp3+ regulatory T cells of psoriasis patients easily differentiate into IL-17A-producing cells and are found in lesional skin. *J. Invest. Dermatol.* 131: 1853–1860.
 25. Koenen, H. J. P. M., R. L. Smeets, P. M. Vink, E. van Rijssen, A. M. H. Boots, and I. Joosten. 2008. Human CD25^{high}Foxp3^{pos} regulatory T cells differentiate into IL-17-producing cells. *Blood* 112: 2340–2352.
 26. Brucklacher-Waldert, V., K. Steinbach, M. Lioznov, M. Kolster, C. Hölscher, and E. Tolosa. 2009. Phenotypical characterization of human Th17 cells unambiguously identified by surface IL-17A expression. *J. Immunol.* 183: 5494–5501.
 27. Ueda, A., L. Zhou, and P. L. Stein. 2012. Fyn promotes Th17 differentiation by regulating the kinetics of ROR γ t and Foxp3 expression. *J. Immunol.* 188: 5247–5256.
 28. Chen, Y., C. J. Haines, I. Gutcher, K. Hochweller, W. M. Blumenschein, T. McClanahan, G. Hämmerling, M. O. Li, D. J. Cua, and M. J. McGeachy. 2011. Foxp3(+) regulatory T cells promote T helper 17 cell development in vivo through regulation of interleukin-2. *Immunity* 34: 409–421.
 29. Marski, M., S. Kandula, J. R. Turner, and C. Abraham. 2005. CD18 is required for optimal development and function of CD4+CD25+ T regulatory cells. *J. Immunol.* 175: 7889–7897.
 30. Horwitz, D. A., S. G. Zheng, and J. D. Gray. 2008. Natural and TGF-beta-induced Foxp3(+)CD4(+) CD25(+) regulatory T cells are not mirror images of each other. *Trends Immunol.* 29: 429–435.
 31. Lebowitz, M. 2003. Psoriasis. *Lancet* 361: 1197–1204.
 32. El-Sayed, Z. A., D. H. El-Ghoneimy, H. Abd-Allah, and H. M. Afifi. 2011. A rare association between leukocyte adhesion deficiency type I and psoriasis in humans. *Allergy Asthma Immunol. Res.* 3: 138–140.
 33. van Pelt, J. P. A., S. H. H. Kuijpers, P. C. M. van de Kerkhof, and E. M. G. J. de Jong. 1998. The CD11b/CD18-integrin in the pathogenesis of psoriasis. *J. Dermatol. Sci.* 16: 135–143.
 34. Talamonti, M., G. Spallone, A. Di Stefani, A. Costanzo, and S. Chimenti. 2011. Efalizumab. *Expert Opin. Drug Saf.* 10: 239–251.
 35. Kess, D., A. K. Lindqvist, T. Peters, H. Wang, J. Zamek, R. Nischt, K. W. Broman, R. Blakytyn, T. Krieg, R. Holmdahl, and K. Scharffetter-Kochanek. 2006. Identification of susceptibility loci for skin disease in a murine psoriasis model. *J. Immunol.* 177: 4612–4619.
 36. Wirmsberger, G., M. Hinterberger, and L. Klein. 2011. Regulatory T-cell differentiation versus clonal deletion of autoreactive thymocytes. *Immunol. Cell Biol.* 89: 45–53.
 37. Gatzka, M. G., R. H. Newton, and C. M. Walsh. 2009. Altered thymic selection and increased autoimmunity caused by ectopic expression of DRAK2 during T cell development. *J. Immunol.* 183: 285–297.
 38. Bachmann, M. F., K. McKall-Faienza, R. Schmits, D. Bouchard, J. Beach, D. E. Speiser, T. W. Mak, and P. S. Ohashi. 1997. Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation. *Immunity* 7: 549–557.
 39. Wuest, T. Y., J. Willette-Brown, S. K. Durum, and A. A. Hurwitz. 2008. The influence of IL-2 family cytokines on activation and function of naturally occurring regulatory T cells. *J. Leukoc. Biol.* 84: 973–980.
 40. Cheng, G., A. Yu, and T. R. Malek. 2011. T-cell tolerance and the multi-functional role of IL-2R signaling in T-regulatory cells. *Immunol. Rev.* 241: 63–76.
 41. Wei, L., A. Laurence, and J. J. O'Shea. 2008. New insights into the roles of Stat5a/b and Stat3 in T cell development and differentiation. *Semin. Cell Dev. Biol.* 19: 394–400.
 42. Lio, C. W., and C. S. Hsieh. 2008. A two-step process for thymic regulatory T cell development. *Immunity* 28: 100–111.
 43. Ziegler, S. F., and J. H. Buckner. 2009. FOXP3 and the regulation of Treg/Th17 differentiation. *Microbes Infect.* 11: 594–598.
 44. Yang, X. O., R. Nurieva, G. J. Martinez, H. S. Kang, Y. Chung, B. P. Pappu, B. Shah, S. H. Chang, K. S. Schluns, S. S. Watowich, et al. 2008. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity* 29: 44–56.
 45. Sather, B. D., P. Treuting, N. Perdue, M. Miazgowiec, J. D. Fontenot, A. Y. Rudensky, and D. J. Campbell. 2007. Altering the distribution of Foxp3(+) regulatory T cells results in tissue-specific inflammatory disease. *J. Exp. Med.* 204: 1335–1347.
 46. Debes, G. F., C. N. Arnold, A. J. Young, S. Krautwald, M. Lipp, J. B. Hay, and E. C. Butcher. 2005. Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. *Nat. Immunol.* 6: 889–894.
 47. Bromley, S. K., T. R. Mempel, and A. D. Luster. 2008. Orchestrating the orchestrators: chemokines in control of T cell traffic. *Nat. Immunol.* 9: 970–980.
 48. Quintana, F. J., A. S. Basso, A. H. Iglesias, T. Korn, M. F. Farez, E. Bettelli, M. Caccamo, M. Oukka, and H. L. Weiner. 2008. Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature* 453: 65–71.
 49. Zhou, L., J. E. Lopes, M. M. Chong, I. I. Ivanov, R. Min, G. D. Vitoria, Y. Shen, J. Du, Y. P. Rubtsov, A. Y. Rudensky, et al. 2008. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgamma γ t function. *Nature* 453: 236–240.
 50. Xu, L., A. Kitani, I. Fuss, and W. Strober. 2007. Cutting edge: regulatory T cells induce CD4⁺CD25⁺Foxp3⁺ T cells or are self-induced to become Th17 cells in the absence of exogenous TGF- β . *J. Immunol.* 178: 6725–6729.

Supplemental Figure S1

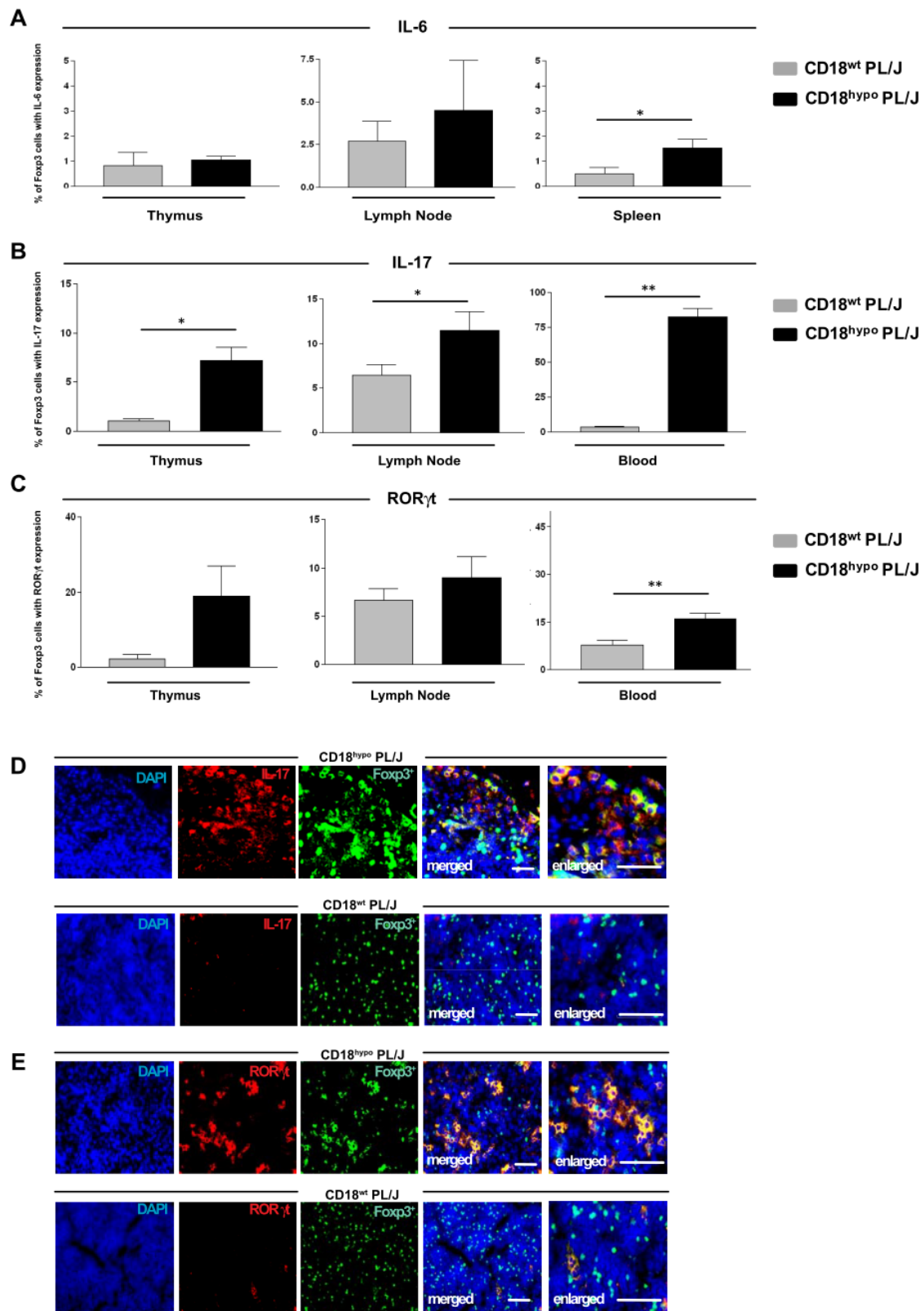


Figure S1. Altered cytokine profiles of peripheral Foxp3⁺ Tregs and systemic inflammatory cytokines may both contribute to increased conversion of peripheral PL/J CD18^{hypo} Tregs into Th17 cells. Expression levels of cytokines IL-6 and IL-17 in Foxp3⁺ Tregs in thymic and secondary lymphoid organs of CD18^{hypo} PL/J mice are variably increased and contribute to an inflammatory environment in affected mice. **A.** Percentages of IL-6 expressing Foxp3⁺ cells are increased in lymph nodes and spleens of affected CD18^{hypo} PL/J (black) compared to CD18^{wt} PL/J mice (light grey) and similar in thymic nTregs as measured by FACS analysis. The experiment was performed on 3 individual CD18^{wt} PL/J and CD18^{hypo} PL/J mice. **B.** Variable increases in the percentage of Foxp3⁺ IL-17 expressing cells were detectable in thymus, lymph nodes and blood of CD18^{hypo} PL/J mice (FACS). In addition, overall IL-17 levels were increased in affected skin and draining lymph nodes (qPCR data not shown here). The experiment was performed on 3 individual mice from CD18^{wt} PL/J and CD18^{hypo} PL/J strains. **C.** Expression level of transcription factor RORγt in Foxp3⁺ Tregs was variably increased in thymi and lymph nodes of CD18^{hypo} PL/J mice compared to CD18^{wt} PL/J mice. A significant increase in RORγt expressing cells was detectable in blood of diseased CD18^{hypo} PL/J mice. This experiment was performed on 3 individual CD18^{wt} PL/J and CD18^{hypo} PL/J mice. **D and E.** Significantly increased numbers of Foxp3⁺IL-17⁺ T cells and RORγt⁺IL-17⁺ cells were observed in inflamed lymph nodes of CD18^{hypo} PL/J mice compared to lymph nodes of healthy CD18^{wt} PL/J mice by immunostaining (Foxp3⁺ T cells, green; IL-17⁺ cells, red; DAPI stained nuclei, blue, original magnification 40x). This experiment was performed in triplicate with 3 individual CD18^{wt} PL/J and CD18^{hypo} PL/J mice, respectively.

Supplemental Figure 2

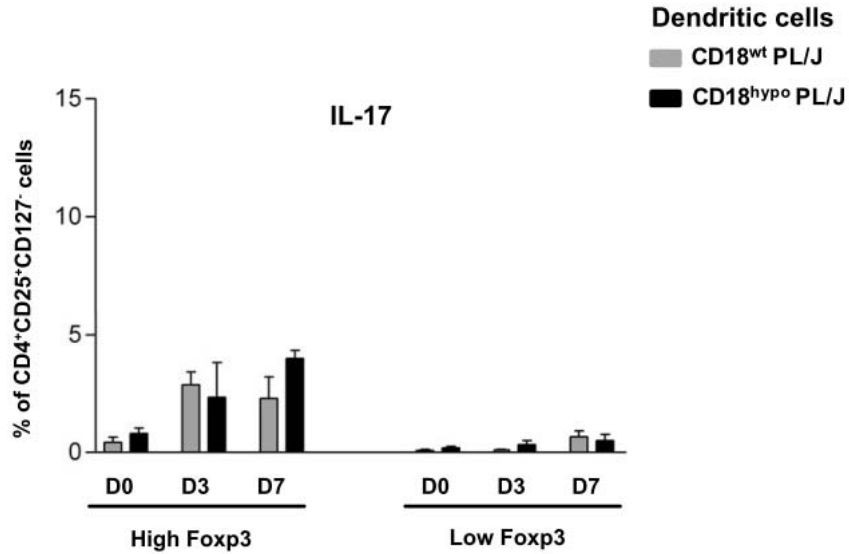


Figure S2. CD18^{hypo} PL/J dendritic cells do not confer significantly altered differentiation of Balb/c Tregs into Th17 cells.

Allogeneic stimulation of Balb/c derived Tregs with DC from either CD18^{hypo} or CD18^{wt} PL/J mice for 7 days did not result in any significant conversion into Th17 cells. Co-culture of Balb/c Tregs with CD18^{hypo} PL/J DC in the presence of IL-2 caused only slightly increased production of IL-17 by Balb/c Tregs in comparison to co-culture with CD18^{wt} PL/J DC. The experiment was performed with DC from 3 individual CD18^{wt} PL/J and CD18^{hypo} PL/J mice, respectively.

Supplemental Figure 3

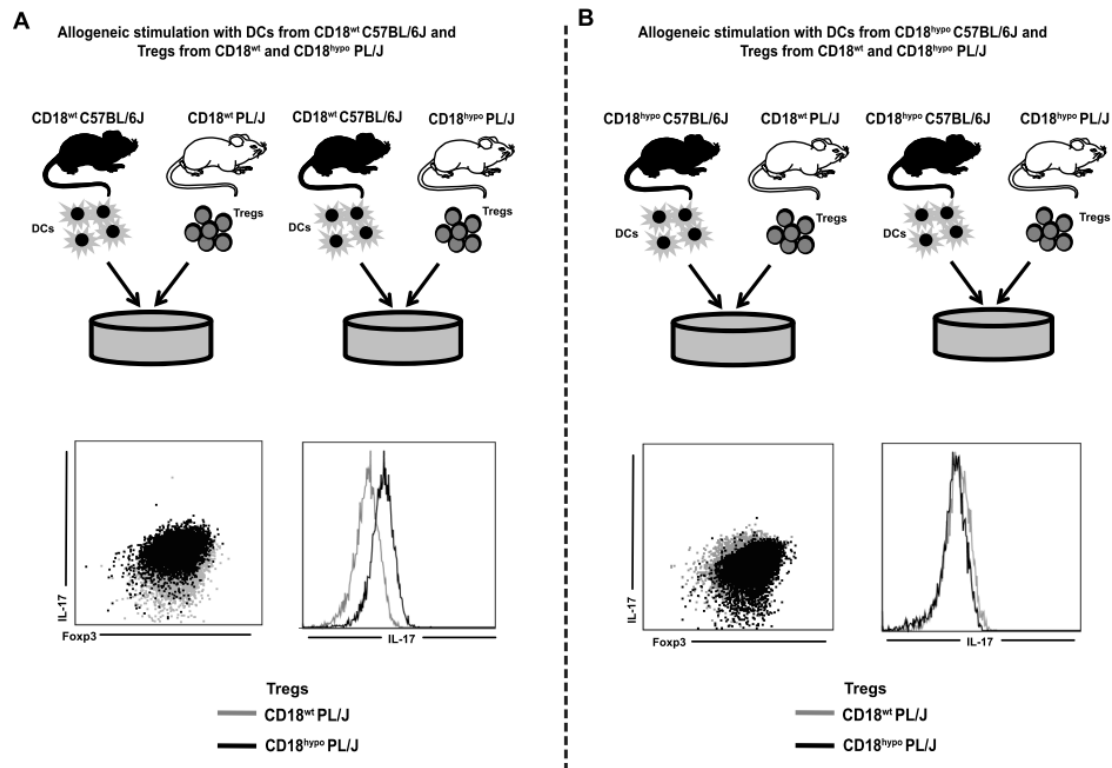


Figure S3. Control assessment for alloreactive activity. As a control readout experiment to assess the levels of alloreactive activity and to investigate the effect of reduced CD18 levels on Tregs versus DCs, allogeneic stimulation of Tregs derived from CD18^{wt} PL/J and CD18^{hypo} PL/J mice (white icons), was performed with DCs from either CD18^{wt} C57BL/6J (A) or from CD18^{hypo} C57BL/6J mice (black icons) (B) as indicated in the graphical illustrations in the upper parts of both panels. **A.** Higher IL-17 expression of Foxp3⁺IL-17⁺ T cells detected on day 7 in co-culture experiments of CD18^{hypo} PL/J Tregs and CD18^{wt} C57BL/6J DCs compared to CD18^{wt} PL/J Tregs co-cultured with CD18^{wt} C57BL/6J DCs supports our hypothesis of a higher tendency of CD18^{hypo} Tregs to differentiate into IL-17

producing cells compared to CD18^{wt} Tregs. **B.** However, co-culture experiments with DCs derived from CD18^{hypo} C57BL/6J and Tregs from CD18^{wt} PL/J/ or CD18^{hypo} PL/J mice, respectively, did not show any significant difference in IL-17 expression of Foxp3⁺IL-17⁺ T cells. This experiment was performed with DC or Tregs from 3 individual CD18^{wt} C57BL/6J and CD18^{hypo} C57BL/6J mice (DC) or CD18^{wt} PL/J and CD18^{hypo} PL/J mice (Tregs), respectively.

Supplemental Figure 4

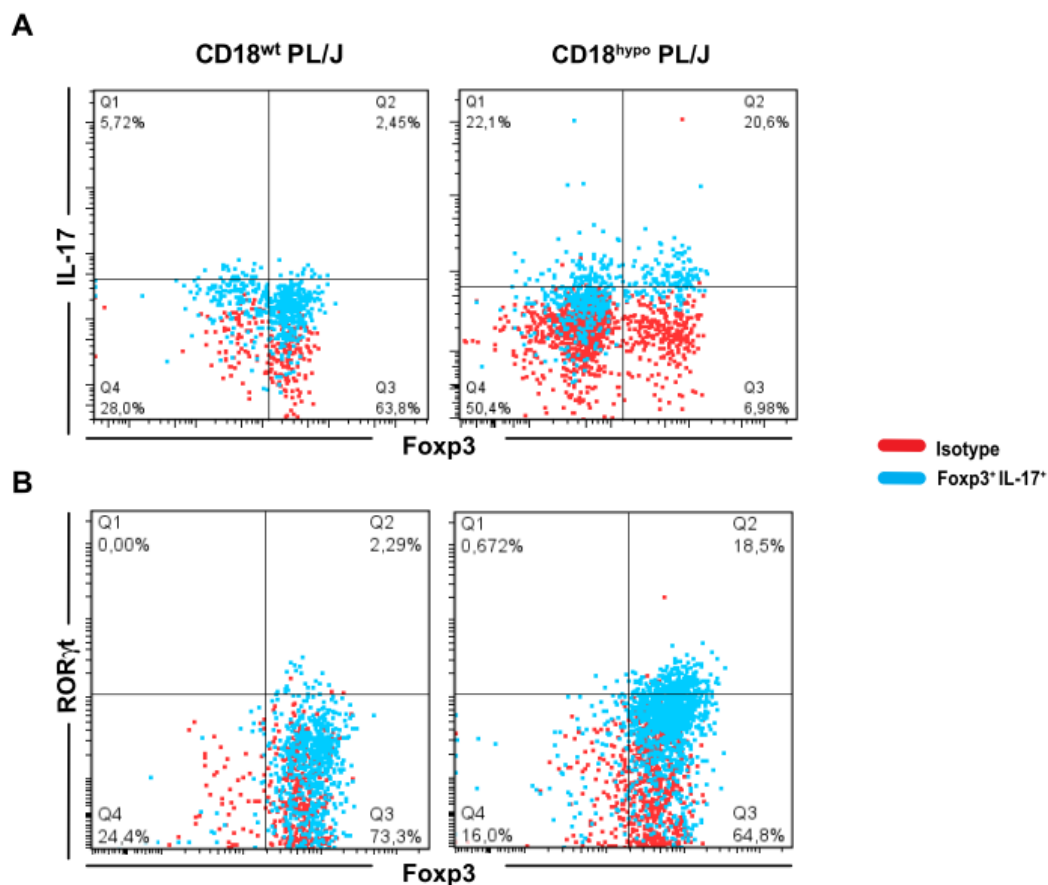


Figure S4. Single-cell resolution evidence of lineage duality. **A.** Representative dot plots for differentiation of Tregs into IL-17 producing Th17 cells in co-culture experiments on day 7. Similar results were reproducible with Tregs from 3 individual CD18^{hypo} or CD18^{wt} PL/J mice, respectively. Red and blue dots represent isotype control IgG and anti-IL-17 stained Foxp3⁺ T cells, respectively. **B.** Representative dot plots for Foxp3⁺RORγ⁺ T cell fractions in DC-Treg co-culture experiments on day 7. Red and blue dots represent isotype control IgG and anti-RORγt stained Foxp3⁺ T cells, respectively. Results were reproducible in 3 individual mice of the CD18^{hypo} or CD18^{wt} PL/J strain, respectively.