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Immunoproteasome Subunit LMP7 Deficiency and Inhibition Suppresses Th1 and Th17 but Enhances Regulatory T Cell Differentiation

Khalid W. Kalim,*,† Michael Basler,*,‡ Christopher J. Kirk,§ and Marcus Groettrup*,†,‡

The immunoproteasome generates peptides presented on MHC class I molecules to cytotoxic T cells. ONX 0914 (formerly called PR-957) is a selective inhibitor of the immunoproteasome subunit low molecular mass polypeptide (LMP) 7 (β5i) that attenuates disease progression in mouse models of diabetes, colitis, and arthritis. The aim of this study was to investigate the effect of LMP7-specific inhibition on major Th cell differentiation pathways involved in the progression of autoimmune diseases in vitro and in vivo. We used ONX 0914-treated wild-type $CD4^+$ T cells and also $LMP7^{-/-}$ $CD4^+$ T cells under different Th cell-polarizing conditions, focusing on the effector cytokines and transcription factors involved, and compared them with wild-type $CD4^+$ T cells. Mouse models of dextran sodium sulfate-induced colitis and a T cell transfer model of colitis were used for in vivo assessment. Deletion or inhibition of LMP7 suppressed generation of Th17 but promoted regulatory T cell (Treg) development. In developing Th17 cells, immunoproteasome inhibition blocked phosphorylation of STAT3, whereas in Tregs, SMAD phosphorylation was enhanced. Additionally, LMP7 inhibition led to reduced STAT1 phosphorylation and Th1 differentiation. These findings were confirmed in vivo as LMP7 inhibition or deficiency resulted in reduced Th1 and Th17 expansion while promoting Treg development in dextran sodium sulfate-induced colitis. Also, in a T cell-dependent transfer model of colitis, LMP7-specific inhibition led to reduced Th1 and Th17 differentiation in vivo. LMP7 governs Th cell lineage determination by affecting the balance of receptor proximal signals during differentiation. These data render LMP7 a promising drug target for the treatment of autoimmune diseases. ***The Journal of Immunology*****, 2012, 189: 4182–4193.**

The proteasome constitutes major proteolytic machinery in the cell, which is responsible for generating antigenic peptides to be presented to cytotoxic T cells by MHC class I molecules (1–3). The 20S core of the 26S constitutive proteasome harbors the three catalytic subunits β1, β2, and β5 in the inner β-rings, which are responsible for caspase-like, trypsin-like, and chymotrypsin-like activities, respectively (4). These constitutive subunits are replaced by the immunoproteasome subunits β1i (low molecular mass polypeptide [LMP] 2), β2i (MECL-1), and β5i (LMP7), respectively, in cells of hematopoietic origin or can be induced in nonhematopoietic cells by the action of the proinflammatory cytokines TNF-α and IFN-γ (5–7). Apart from its role in Ag presentation, immunoproteasomes shape the T cell repertoire and are responsible for the survival and expansion of T cells (8–11). We have previously characterized a cell-permeable epoxyketone immunoproteasome inhibitor called ONX 0914 (formerly named PR-957) that selectively inhibits β5i (LMP7) in human and mouse cells (12). ONX 0914 blocked the production of proinflammatory cytokines from human PBMCs and activated mouse splenocytes, and it also inhibited IL-17–producing T cells under Th17-polarizing cytokines in vitro (12). Because ONX 0914 treatment resulted in an attenuation of disease progression in experimental diabetes, arthritis, and colitis (12, 13), we decided to investigate the molecular effects of LMP7-specific inhibition on different Th cell differentiation signaling pathways important in regulating autoimmunity.

Naive $CD4^+$ T cells can differentiate into different Th cell lineages depending on the cytokines in the microenvironment. Initially, two effector Th subsets were distinguished: Th1 and Th2 (14). Th1 differentiation is promoted by IL-12 and transcription factors STAT1 and STAT4 along with T-bet (15). Th2 differentiation is driven by IL-4 and the transcription factors GATA-3 and STAT6 (16). Whereas Th1 cells produce IFN-γ and are responsible for clearing intracellular pathogens, Th2 cells produce IL-4 as the signature cytokine and help to clear extracellular pathogens (14, 17). Subsequently, Th17 cells were identified as mediators of autoimmune diseases like arthritis, multiple sclerosis, psoriasis, and inflammatory bowel disease (18–22). TGF-β in combination with IL-6 drive the differentiation of Th17 cells, and IL-23 is required for terminal differentiation of Th17 cells into mature effector cells (23–25). Retinoic acid-related orphan receptor (ROR)γt is a Th17-specific transcription factor (23, 26), and STAT3 plays an important role in Th17 differentiation via induction of RORγt and RORα (23). Regulatory T cells (Tregs) are involved in suppressing overactivated T cells and maintaining

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K.W.K. designed and performed the experiments and wrote the manuscript; M.B. and C.J.K. provided technical expertise and reagents and corrected the manuscript; and M.G. designed the experiments, supervised the project, and refined the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: DSS, dextran sodium sulfate; LMP, low molecular mass polypeptide; mLN, mesenteric lymph node; PIAS, protein inhibitor of activated Stat; ROR, retinoic acid-related orphan receptor; Treg, regulatory T cell; wt, wild-type.

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immune homeostasis (27). Their differentiation is driven by the transcription factor Foxp3 and induced by TGF-β (27, 28). Immunosuppressive Treg and proinflammatory Th17 cells can be interconverted and are reciprocally regulated during differentiation (29).

We report in this study, using both pharmacologic and genetic deletion of LMP7, the role of the immunoproteasome in the differentiation and function of multiple Th lineages and investigated how LMP7 deletion affects transcriptional regulators and signaling pathways involved in Th lineage determination. We show that LMP7-specific inhibition blocks Th1 differentiation both in vitro and in vivo without affecting Th2 differentiation. In addition, it shifts the plastic equilibrium between Th17 and Tregs toward the latter by inhibiting the phosphorylation of STAT3. These findings provide a likely mechanistic basis for the attenuation of autoimmune diseases by LMP7 inhibition.

Materials and Methods

Mice and reagents

C57BL/6 mice (H-2^b) were purchased from the animal facility of the University of Constance. LMP7$^{-/-}$ gene-targeted mice were provided by J. Monaco (Department of Molecular Genetics, Cincinnati Medical Center, Cincinnati, OH). RAG2$^{-/-}$ mice were obtained from Dr. Maries van den Broek, Zürich University (Zurich, Switzerland) Anti-CD3 (17A2), anti-IL-4 (11B11), anti–IFN-γ (XMG1.2), PE–anti-RORγt (AFKJS-9), allophycocyanin–anti–IL-17A (17B7), Alexa Fluor 647 anti-mouse gp130, eFluor 450 anti-mouse Foxp3 (FJK-16s), eFluor 450 fixable viability dye, and rIL-6 were purchased from eBioscience (Natutec). TGF-β1 was from PeproTech. rIL-12, allophycocyanin anti-mouse IL-4, PE anti-mouse CD25 (3C7), FITC anti-mouse IFN-γ, mouse IL-17 ELISA kit, and mouse IFN-γ ELISA kit were all purchased from BD Biosciences. MG132 was from Sigma-Aldrich. Western blot anti-mouse Abs for pSTAT3, STAT3, pSTAT1, pSTAT4, pSTAT6, pSMAD2, and pSMAD3 were all purchased from Cell Signaling Technology. Anti-mouse actin Ab was from Santa Cruz Biotechnology. Animal Studies were carried out in accordance with the animal research review board of Regierungspräsidium Freiburg.

Th cell differentiation

CD4$^+$ T cells from spleens of 6–8-wk-old mice were purified by anti-CD4 (L3T4) microbeads and magnetic separation (Miltenyi Biotec). Purified cells were activated with plate-bound anti-CD3 and anti-CD28 for 3 d plus cytokines and neutralizing Abs for the desired polarization as follows: IL-12 (10 ng/ml) and anti–IL-4 (10 μg/ml) for Th1 polarization; IL-4 (50 ng/ml), IL-2 (200 μg/ml), and anti–IL-12 for Th2 polarization; TGF-β (2.5 ng/ml), IL-6 (30 ng/ml), anti–IFN-γ (10 μg/ml), and anti–IL-4 (10 μg/ml) for Th17 polarization; and TGF-β (5 ng/ml), IL-2 (200 μg/ml), and anti–IFN-γ (10 μg/ml) for Treg polarization.

Intracellular staining and flow cytometry

Polarized CD4$^+$ T cells were restimulated for 4 h with PMA (5 ng/ml) and ionomycin (200 ng/ml) along with brefeldin A (Sigma-Aldrich). After surface staining with indicated Abs, cells were fixed with Fixation/Permeabilization Buffer (eBioscience), made permeable with Permeabilization Buffer (eBioscience), and stained with fluorescence-linked Abs before analysis on FACSAria or Accuri C6 flow cytometers (BD Biosciences). Events were recorded and analyzed with FlowJo software (Tree Star).

Quantitative real-time RT-PCR

Total RNA was extracted (Nucleospin RNA isolation kit; Macherey-Nagel) from the polarized CD4$^+$ T cells stimulated for indicated time points followed by reverse transcription (Promega reverse transcription kit; Promega). The cDNA was used for PCR amplification using the light cycler instrument (Roche) and a SYBR Green I-based method with the following primers: IL-17A forward, 5′-CTCCAGAAGGCCCTCAGACTAC-3′; IL-17A reverse, 5′-GCTTTCCCTCCGCATTGACACAG-3′; RORγt forward, 5′-GTGGACTTCGTTTGAGGAAAC-3′; RORγt reverse, 5′-ACTTCCTCTGGTAGCTGGTCAC-3′; IL-4 forward, 5′-CATCGGCATTTTGAAAGAG-3′; IL-4 reverse, 5′-CGAGCTCACTCTCTCTGTGGTG-3′; GATA-3 forward, 5′-CTGGAGGAGGAACGCTAATG-3′; and GATA-3 reverse, 5′-AGATGTGGCTCAGGGATGAC-3′.

The quantitative value of each sample was normalized to hypoxanthine phosphoribosyltransferase or 18S rRNA, which were used as reference genes.

Immunoblot analysis

Purified CD4$^+$ T cells were cultured for respective time periods, and cellular extracts were prepared using complete lysis buffer (Tris, NaCl, Triton X-100, SDS, and protease inhibitor mixture). Protein quantification was performed with cellular extracts using protein quantification reagents (Bio-Rad). Equal amounts of protein extracts were separated by SDS-PAGE and transferred to nitrocellulose membrane (Whatman Protran). Membranes were probed with Abs indicated in the figures. β-actin was used as loading control.

Dextran sodium sulfate-induced colitis

Colitis was induced in 8–10-wk-old female mice by adding 2.2% dextran sodium sulfate (DSS) (m.w. 36,000–50,000; MP Biomedicals, Solon, OH) to the drinking water, beginning on day 0 for 5 d; thereafter, they were given regular drinking water. We performed daily measurements of body weight throughout the experiment.

Adoptive transfer model of colitis

A total of 5×10^5 magnetically purified CD4$^+$CD62L$^+$ naive T cells (Miltenyi Biotec) were injected i.p. into age- and sex-matched RAG2$^{-/-}$ mice. One group of the mice was treated with PBS and the other with the LMP7-specific inhibitor ONX 0914 every alternate day for 2 wk. Mice were sacrificed thereafter, and spleen and mesenteric lymph nodes (mLN) were isolated for flow cytometric analysis.

Treatment of mice with ONX 0914

ONX 0914 was formulated in aqueous solution of 10% (w/v) sulfobutylether-β-cyclodextrin and 10 mM sodium citrate (pH 6) and administered to mice as an s.c. dose of 10 mg/kg (in a volume of 100 μl).

Isolation of lamina propria lymphocytes

The colon was removed, cut into smaller pieces, and washed in HBSS and DTT. Intraepithelial mononuclear cells were removed by washing the cells in HBSS along with EDTA. The remaining tissue was digested at 37°C with collagenase type IV from Sigma-Aldrich (0.5 mg/ml). The cell suspension was filtered and washed. Lamina propria lymphocytes were purified over a gradient of 40–80% Percoll and isolated from the interface layer and washed twice in media before restimulation with anti-CD3/CD28 for 6 h along with brefeldin A (for the last 5 h), followed by intracellular staining as described above.

Statistical analysis

The statistical significance of the differences was determined using the Student t test. GraphPad Prism software (version 4.03) (GraphPad, San Diego, CA) was used for performing all statistical analyses. Statistical significance was achieved when $p < 0.05$.

Results

Specific inhibition of immunoproteasome subunit LMP7 downregulates RORγt through reduced phosphorylation of STAT3 in vitro

LMP7 inhibition started to downregulate IL-17 mRNA after 24 and 48 h of Th17-polarizing conditions, as there was a ~2-fold reduction in IL-17 mRNA (Fig. 1 *A*). Interestingly, RORγt mRNA was downregulated quite early in Th17-polarizing conditions by LMP7 specific inhibition. There was a ~5-fold decrease in RORγt mRNA in ONX 0914-treated cells after 12 h of Th17-polarizing conditions. We also looked at RORγt by intracellular staining after 48 h of Th17-polarizing conditions (Fig. 1B). There was a ~10-fold induction of RORγt when the cells were polarized under Th17 conditions in comparison with Th0 conditions. CD4$^+$ T cells that were Th17 polarized in the presence of ONX 0914 showed a ~4-fold downregulation of RORγt in comparison with untreated cells, and this correlated with the downregulation of RORγt mRNA.

To investigate whether LMP7-specific inhibition was affecting the phosphorylation of STAT3 in CD4$^+$ T cells, we stimulated purified CD4$^+$ T cells overnight with anti-CD3/CD28 in the presence or absence of 200 nM ONX 0914. On the next day, we added TGF-β plus IL-6 and harvested the cells after the indicated

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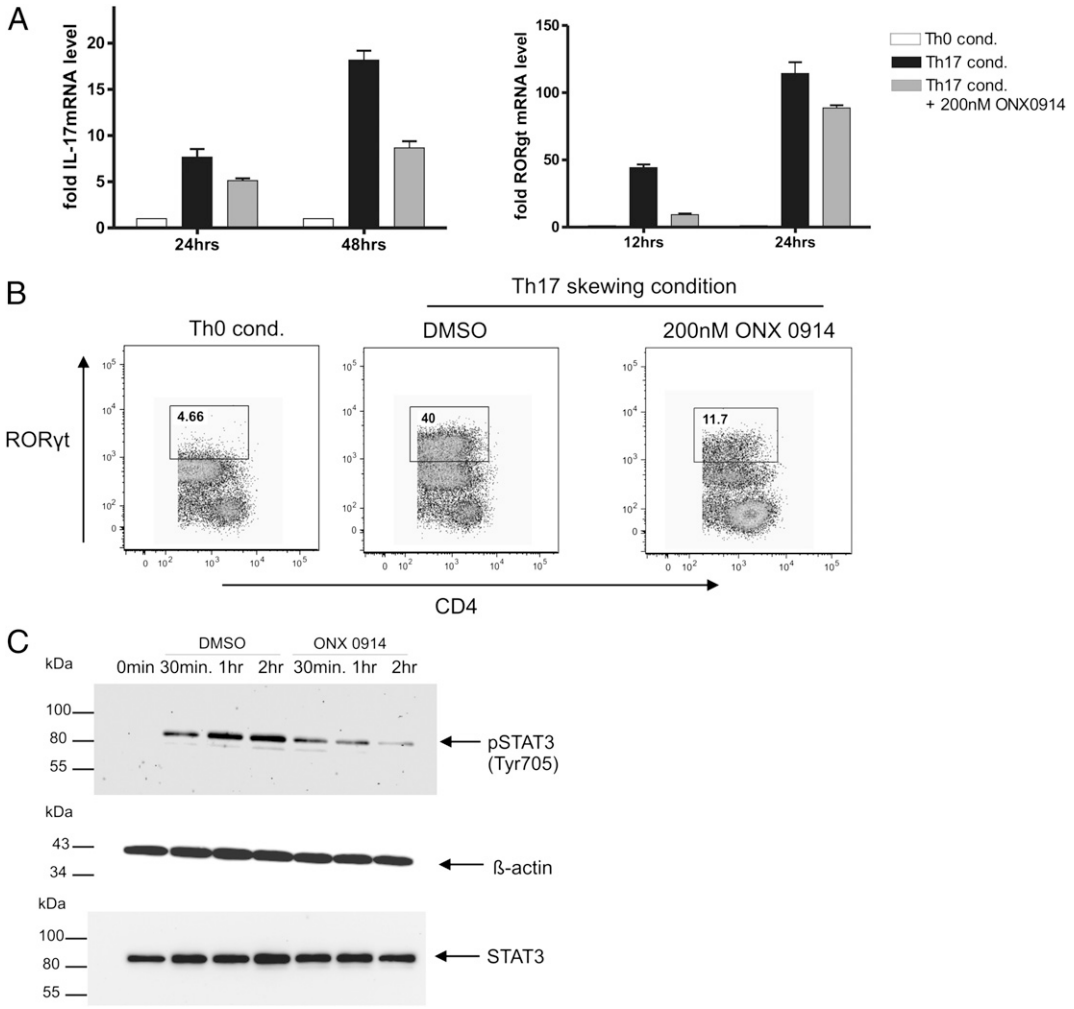

FIGURE 1. Specific inhibition of LMP7 downregulates RORγt through reduced phosphorylation of STAT3. (**A**) Quantitative RT-PCR analysis of IL-17 and RORγt mRNAs isolated from magnetically purified CD4$^+$ T cells from spleens of C57BL/6 mice cultured for indicated time periods under Th0- and Th17-polarizing conditions in the presence or absence of 200 nM ONX 0914. 18S rRNA was used as the reference gene. (**B**) Flow cytometric analysis of magnetically purified CD4$^+$ T cells from spleen of C57BL/6 mice cultured under Th17-polarizing conditions for 48 h. (**C**) Western blot analysis of purified CD4$^+$ T cells that were cultured overnight with anti-CD3/CD28 in the presence or absence of 200 nM ONX 0914 and then stimulated with TGF-β and IL-6 for indicated time periods. Data are representative of at least three independent experiments.

time points. We found an increased phosphorylation of STAT3 after TGF-β plus IL-6 addition to the cells (Fig. 1C). There was less phospho-STAT3 in the ONX 0914-treated CD4$^+$ T cells in comparison with untreated cells after 1 and 2 h of TGF-β plus IL-6 stimulation without a corresponding change in total STAT3 levels (Fig. 1C). An analysis of IL-6R expression by flow cytometry showed no difference in gp130 (IL-6R) expression between the untreated or ONX 0914-treated CD4$^+$ T cells activated overnight with anti-CD3/CD28 (Supplemental Fig. 1). To find out whether the reduced phosphorylation of STAT3 was due to an increased degradation of phosphorylated STAT3 by the ubiquitin-proteasome system, we activated purified CD4$^+$ T cells with anti-CD3/CD28 in the presence or absence of ONX 0914, and on the next day, we added the broad-spectrum proteasome inhibitor MG132 for 2 h to the cells before TGF-β plus IL-6 stimulation. Western blot analysis of the samples harvested after 1 and 2 h of TGF-β/IL-6 stimulation showed increased phosphorylation of STAT3. CD4$^+$ T cells that were treated only with ONX 0914 showed reduced p-STAT3 as before. Also, samples that were treated only with constitutive proteasome inhibitor MG132 showed less p-STAT3. We could not detect any restoration of p-STAT3 in the samples that were treated with both ONX 0914 and MG132, indicating that the reduced phosphorylation was not due to enhanced degradation by the proteasome (Supplemental Fig. 2).

LMP7-specific inhibition modulates Th17/Treg plasticity in vitro

TGF-β induces both RORγt and Foxp3 in naive CD4$^+$ T cells. Foxp3 binds to RORγt and inhibits its activity, but in the presence of IL-6, this Foxp3-mediated repression of RORγt is relieved, and the cells acquire a Th17 phenotype. LMP7$^{-/-}$ CD4$^+$ T cells showed less IL-17A$^+$ cells and more Foxp3$^+$ cells than wild-type (wt) CD4$^+$ T cells after 3 d of Th17-polarizing conditions (Fig. 2A). Analysis of the supernatants from the Th0- and Th17-polarized wt and LMP7$^{-/-}$ CD4$^+$ T cells revealed that the latter had a ~3-fold lower IL-17A concentration than wt CD4$^+$ T cells (Fig. 2D). Purified CD4$^+$ T cells from C57BL/6 mice were cultured in the presence or absence of 200 nM ONX 0914 under Th17-skewing conditions along with rIL-2 for 24, 48, and 72 h. CD4$^+$ T cells that were cultured in the presence of ONX 0914 showed less IL-17A$^+$ and more Foxp3$^+$ cells than the control cells after 48 and 72 h of Th17-polarizing conditions, which can be attributed to altered IL-6 signaling and reduced phosphorylation of STAT3 by ONX 0914 treatment (Fig. 2B). Because the plasticity

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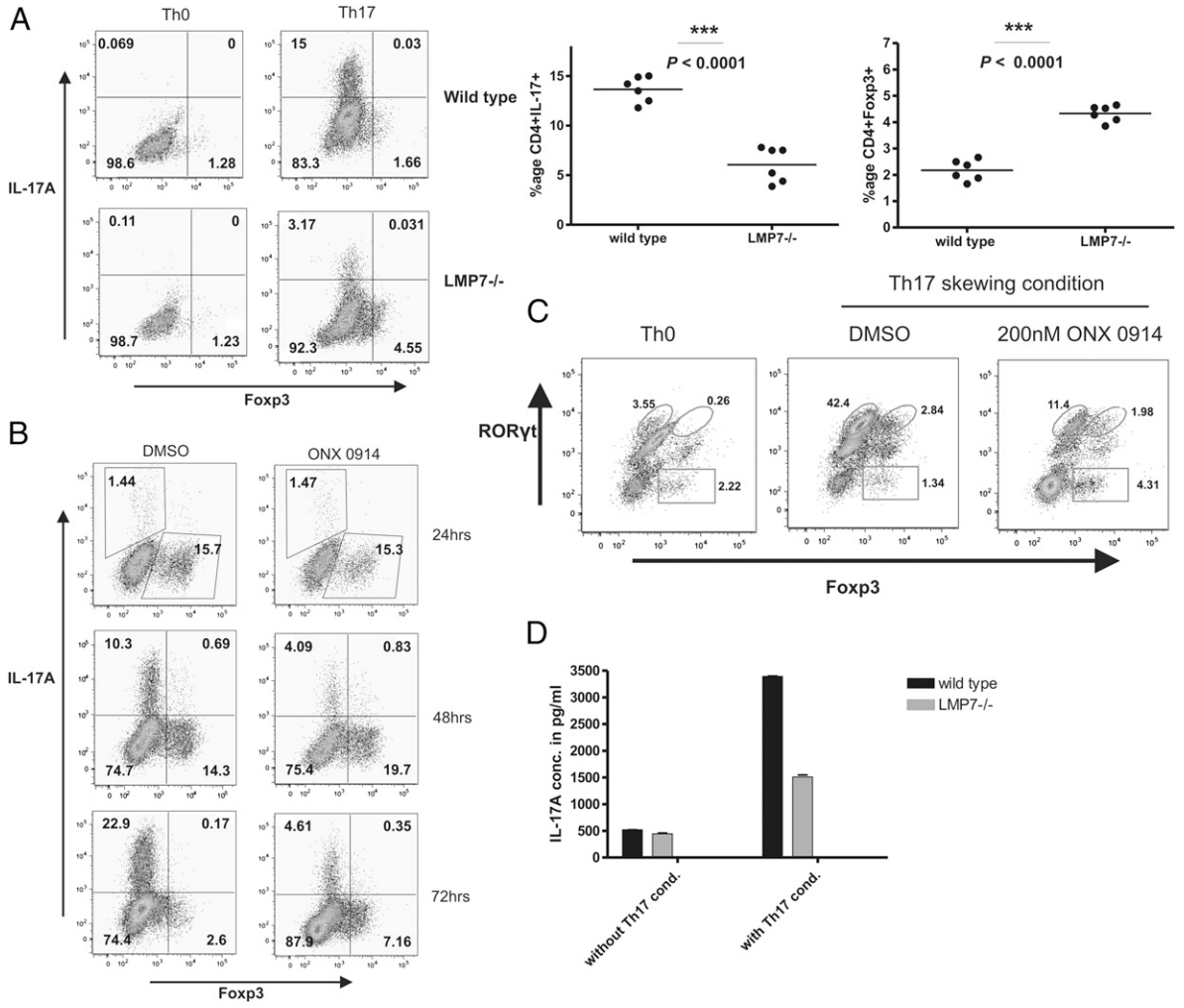

FIGURE 2. LMP7-specific inhibition modulates Th17/Treg plasticity in vitro. (**A**) Flow cytometric analysis of magnetically sorted $CD4^+$ T cells from spleens of wt and $LMP7^{-/-}$ mice, which were activated with anti-CD3/CD28 under Th0- and Th17-polarizing conditions for 72 h, restimulated with PMA and ionomycin in the presence of brefeldin A for 4 h, and then stained for IL-17 and Foxp3. Each symbol in the graph represents an individual mouse, and the horizontal line indicates the mean. (**B**) Flow cytometry of magnetically purified $CD4^+$ T cells from the spleen of C57BL/6 mice cultured under Th17 conditions for 24, 48, and 72 h in the presence or absence of 200 nM ONX 0914. (**C**) Flow cytometric analysis of magnetically purified $CD4^+$ T cells from spleens of C57BL/6 mice cultured under Th17 conditions for 72 h. (**D**) ELISA of culture supernatant from (A); values are normalized to viable cells in the sample after 72 h of culture. Error bars represent triplicate readings in the same experiment. Data are representative of at least three independent experiments.

between Th17 and Tregs occurs at the level of interaction between RORγt and Foxp3, we decided to look at RORγt and $Foxp3^+$ cells by costaining under Th17-polarizing conditions. $CD4^+$ T cells cultured in the presence of ONX 0914 had less RORγt and more Foxp3-producing cells than the untreated cells under Th17-polarizing conditions (Fig. 2C, 2D). Taken together, these data support a role for the immunoproteasome in controlling the balance of Th17/Treg differentiation through the potentiation of STAT3 activation.

Specific inhibition of LMP7 results in reduced Th1 differentiation without any effect on Th2 differentiation in vitro

$CD4^+$ T cells cultured in the presence of ONX 0914 produced 2-fold less IFN-γ–producing cells than the untreated sample under Th1-skewing conditions (Fig. 3A). Also, $LMP7^{-/-}$ $CD4^+$ T cells showed less IFN-γ^+ cells than wt cells under Th1 conditions (Fig. 3B). Because STAT1 governs T-bet expression and Th1 differentiation, we investigated the effect of LMP7 inhibition on the phosphorylation status of STAT1. Naive $CD4^+$ T cells were pulsed for 2 h with ONX 0914 and cultured with IL-12. There was an increased phosphorylation of STAT1 and STAT4 over time. Samples pulsed with ONX 0914 showed less phosphorylation of STAT1 than the untreated control, but no effect on STAT4 was detected (Fig. 3C). ONX 0914 treatment did not alter the number of IL-4^+ cells under Th2-polarizing conditions (Fig. 3D). We also quantitated IL-4 and GATA-3 mRNAs by quantitative RT-PCR analysis and again found no effect of LMP7-specific inhibition (Fig. 3F). ONX 0914 treatment did not affect phosphorylation of STAT6 after 2 h of culture with IL-4 (Fig. 3E).

LMP7-specific inhibition promotes $Foxp3^+$ Tregs with suppressive activity

$CD4^+$ T cells pulsed for 2 h with ONX 0914 and cultured for 3 d under Treg-polarizing conditions showed a >2-fold upregulation of $Foxp3^+$ cells in comparison with untreated cells (Fig. 4A). Because SMAD proteins, particularly SMAD2 and SMAD3, are mainly responsible for driving Foxp3 expression in induced Tregs, we looked for the phosphorylation status of these proteins in the same samples. $CD4^+$ T cells pulsed with ONX 0914 showed more phosphorylated SMAD2 and SMAD3 than the untreated

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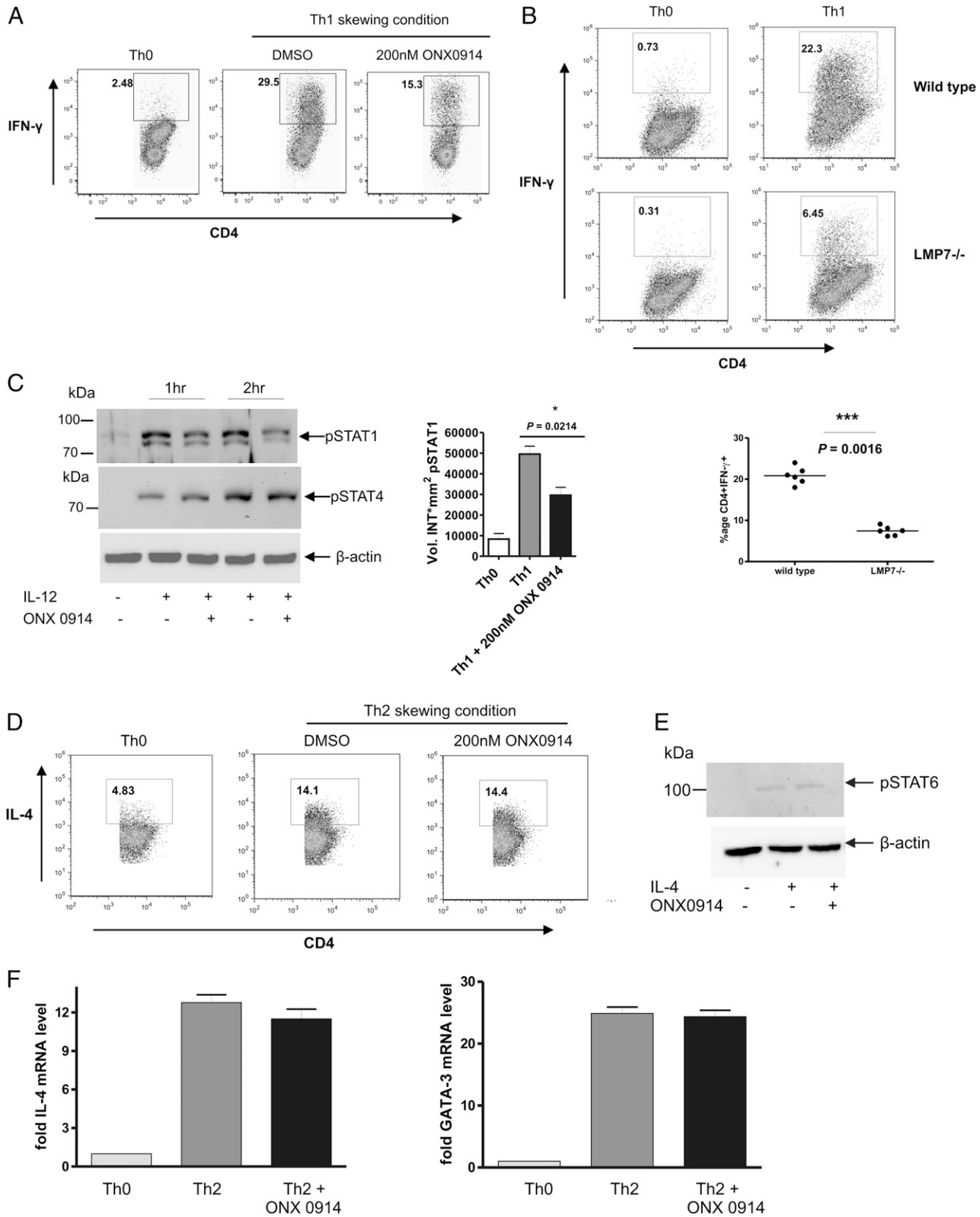

FIGURE 3. LMP7-specific inhibition leads to reduced Th1 differentiation without any effect on Th2 differentiation in vitro. Flow cytometry analysis of magnetically purified $CD4^+$ T cells from spleen of C57BL/6 mice pulsed for 2 h in the presence or absence of 200 nM ONX 0914 and cultured under Th0-polarizing and either under Th1-polarizing (**A**) or Th2-polarizing conditions (**D**) for 72 h, restimulated with PMA and ionomycin in the presence of brefeldin A for 4 h, and stained for IFN-γ and IL-4 for Th1- and Th2-differentiated cells, respectively. (**B**) Flow cytometry analysis of magnetically purified $CD4^+$ T cells from spleen of wt and $LMP7^{-/-}$ mice cultured and restimulated as in (A). Each symbol in the graph represents an individual mouse, and the horizontal line indicates the mean. Western blot analysis of purified $CD4^+$ T cells pulsed for 2 h in the presence or absence of 200 nM ONX 0914 and cultured with IL-12 for 1 and 2 h (**C**) and IL-4 (**E**). The blots were probed with p-STAT1 and p-STAT4 Abs in (C) and p-STAT6 Ab in (E). Actin was used as loading control. Densitometry analysis of p-STAT1 Western blot from three independent experiments are shown. (**F**) Real-time RT-PCR analysis of IL-4 and GATA-3 of RNA isolated from magnetically purified $CD4^+$ T cells from spleens of C57BL/6 mice pulsed for 2 h in the presence or absence of 200 nM ONX 0914 and cultured under Th0- and Th2-polarizing conditions as in (D). 18S rRNA was used as the reference gene. Data are representative of at least three independent experiments.

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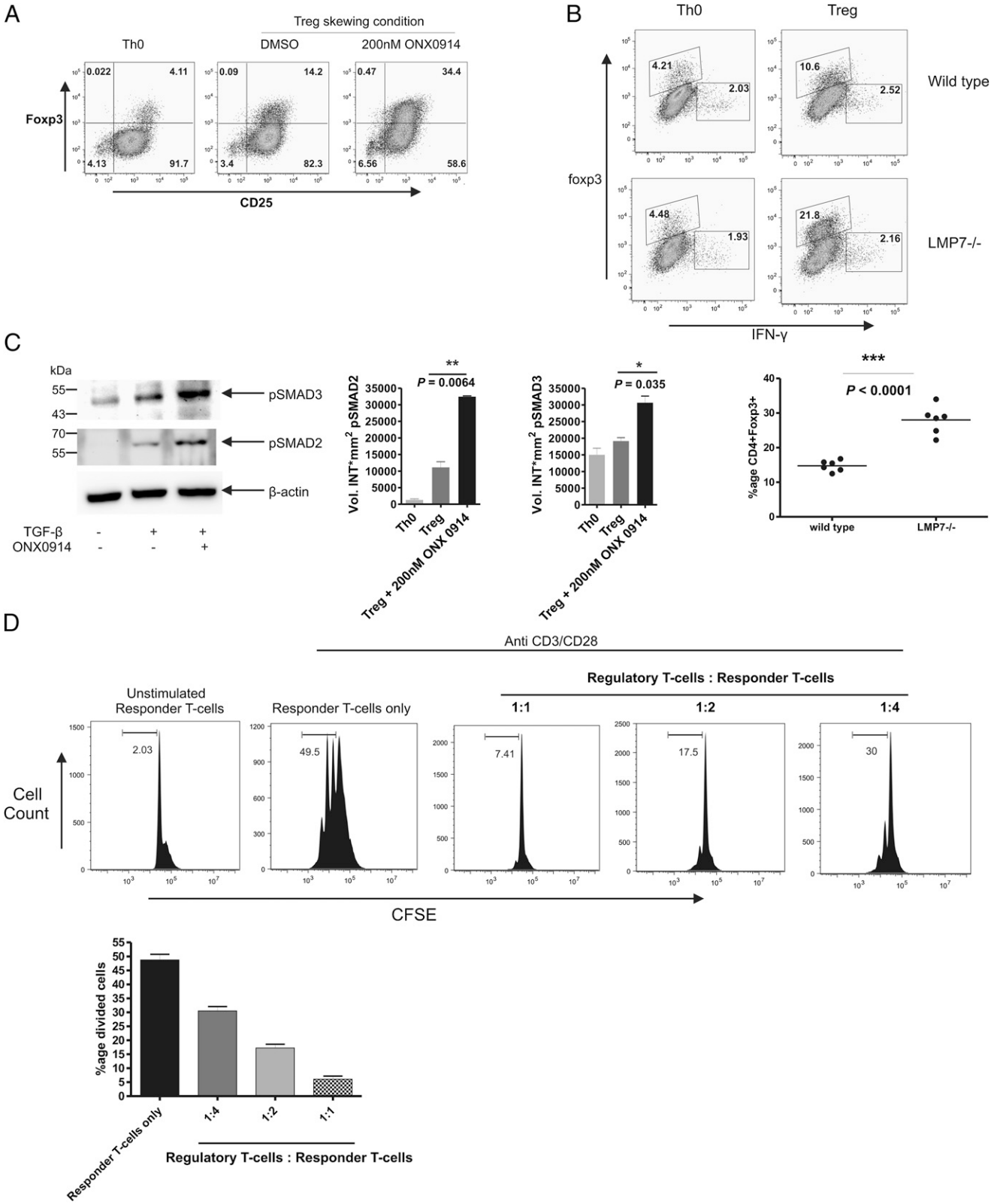

FIGURE 4. LMP7-specific inhibition promotes Foxp3$^+$ Tregs with suppressive activity in vitro through increased phosphorylation of SMAD2 and SMAD3. (**A**) Flow cytometric analysis of magnetically purified CD4$^+$ T cells from spleen of C57BL/6 mice pulsed for 2 h in the presence or absence of 200 nM ONX 0914 and cultured under Th0- and Treg-polarizing conditions for 72 h and stained. (**B**) Flow cytometry analysis of magnetically purified CD4$^+$ T cells from spleen of wt and LMP7$^{-/-}$ mice cultured under Th0 and Treg conditions as in (A) and stained. Each symbol in the graph represents an individual mouse, and small horizontal line indicates the mean. (**C**) Western blot analysis of the cells in (A) with p-SMAD2 and p-SMAD3 Abs after 4-h restimulation with PMA and ionomycin. Actin was used as loading control. Densitometry analysis of p-SMAD2 and p-SMAD3 Western blot from three independent experiments is shown. (**D**) CFSE-labeled responder T cells in increasing concentration were stimulated with anti-CD3/CD28 and incubated with sorted CD4$^+$CD25$^+$ Tregs that were generated with 2 h of pulsing with ONX 0914 under Treg conditions. Proliferation of responder T cells was monitored via dilution of CFSE. Data are representative of at least three independent experiments.

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cells, suggesting that the upregulation of Foxp3 in ONX 0914-treated cells was due to increased phosphorylation of SMAD2 and SMAD3 (Fig. 4C). LMP7$^{-/-}$ CD4$^+$ T cells also showed a >2-fold upregulation of Foxp3$^+$ Tregs compared with wt CD4$^+$ T cells under the same conditions (Fig. 4B). We further wanted to assess the suppressive activity of Tregs generated in the presence of ONX 0914. Therefore, we differentiated purified naive CD4$^+$ T cells, pulsed them with ONX 0914 under Treg-skewing conditions for 3 d, and then sorted CD4$^+$CD25$^+$ Tregs from the culture by FACS. We incubated them with increasing concentrations of CFSE-labeled responder T cells, stimulated them with anti-CD3/CD28, and measured their proliferation. There was a significant suppression of responder T cells by the Tregs even when employed at a ratio of 1:4 (Fig. 4D). We found no difference in the suppressive capacity of the Tregs generated in the presence or absence of ONX 0914 on a single-cell basis at all three dilutions, whereas the purity of sorted CD4$^+$CD25$^+$Tregs from both samples (ONX 0914-treated or untreated) was equal (Supplemental Fig. 3).

Specific inhibition of LMP7 blocks Th1 and Th17 expansion, whereas it promoted Treg differentiation in experimental colitis in vivo

LMP7-specific inhibition has already been shown to protect mice from DSS-induced colitis (13, 30). To assess the effect of LMP7 inhibition on Th1, Th17, and Treg differentiation in this in vivo model, wt and LMP7$^{-/-}$ mice were treated with 2.2% DSS in the drinking water for 5 d, monitored for weight loss, and sacrificed on day 9. Upon DSS treatment, wt mice started to lose weight after 5 d in contrast to LMP7$^{-/-}$ mice, which continued to gain weight (Fig. 5A). LMP7$^{-/-}$ mice had a significantly lower frequency of Th1 and Th17 cells in the lamina propria than wt mice (Fig. 5B). Also, the DSS-fed mice that were treated with ONX 0914 did not show any weight loss in contrast to DSS-treated control mice (Fig. 5C). Flow cytometric analysis of lamina propria lymphocytes showed significantly less Th1 and Th17 cells in the ONX 0914-treated mice in comparison with the PBS-treated control mice (Fig. 5D). Clearly, there were significantly more Foxp3$^+$ Tregs in the mLN of ONX 0914-treated mice in comparison with the PBS-treated control groups after 6 d of DSS treatment (Fig. 5E), thus confirming our in vitro data in this in vivo model of an autoimmune disease.

LMP7-specific inhibition leads to reduced Th1 and Th17 differentiation in a T cell transfer model of colitis in vivo

Magnetically sorted CD4$^+$CD62L$^+$ naive T cells were transferred into RAG2$^{-/-}$ mice and were treated with either PBS or ONX 0914 every alternate day. Mice were sacrificed after 2 wk, and cells from spleen and mLN were analyzed for IFN-γ–producing Th1 cells and IL-17–producing Th17 cells. Flow cytometric analysis showed a significant reduction in the frequency of Th1 cells in the ONX 0914-treated mice as compared with the PBS-treated control groups in both the spleen and mLN (Fig. 6A). ONX 0914-treated mice also showed a lower frequency of IL-17–producing Th17 cells in comparison with the control group (Fig. 6B). ONX 0914-treated mice had significantly lower absolute numbers of Th1 and Th17 cells in the mLN in comparison with the control group (Fig. 6C).

Immunoproteasome subunit LMP7-specific inhibition in Th cells is responsible for reduced Th1 differentiation and enhanced regulatory T cell differentiation in vivo

To investigate whether the immunoproteasome subunit LMP7 inhibition specifically in Th cells is responsible for these effects on different Th cell lineages, we transferred magnetically sorted CD4$^+$ CD62L$^+$ naive T cells from wt mice and also from LMP7$^{-/-}$ mice into RAG2$^{-/-}$ mice. We sacrificed the mice 2 wk after transfer and looked at the Th1 and Tregs from wt and LMP7$^{-/-}$ mice in the spleen and mLN of RAG2$^{-/-}$ mice. There were significantly less Th1 cells in the spleen and mLN of the mice, which received LMP7$^{-/-}$ cells in comparison with the mice that received wt cells (Fig. 7A). Moreover, there was a significant enhancement of the Foxp3$^+$ Tregs in the spleen of mice, which received LMP7$^{-/-}$ cells in comparison with the mice that received wt cells (Fig. 7B). These data supports our in vitro finding with the sorted CD4$^+$ T cells that it is the LMP7-specific inhibition in the Th cells that is responsible for the reported effects on Th cell differentiation.

Discussion

The clinical relevance of the broad-spectrum proteasome inhibitor bortezomib has been limited to the treatment of cancer due to the side effects of the drug (31). Immunoproteasomes are important for the survival and expansion of T cells in a proinflammatory environment (11) that led to the hypothesis that the immunoproteasome subunits might serve as a drug target for suppressing overactivated T cells found predominantly in an autoimmune condition. Indeed, the treatment with the LMP7-selective inhibitor ONX 0914 blocked disease progression in experimental arthritis and colitis (12, 13). In this study, we have looked at the effect of LMP7-specific inhibition at the molecular level focusing on the transcription factors governing Th lineage decisions.

LMP7 regulated the production of IL-17 and RORγt both at the protein and transcriptional level. LMP7-specific inhibition blocked the production of RORγt mRNA early in Th17 differentiation, which points toward an immediate role of LMP7 in the generation of autoreactive T cells. The inhibition of RORγt was most likely due to reduced phosphorylation of STAT3 as a result of LMP7 inhibition. IL-6–induced p-STAT3 inhibits the conversion of naive T cells to Tregs, and it plays a crucial role in regulating the plasticity between Th17 and Tregs (29, 32). There was an increase in Foxp3$^+$ Tregs upon LMP7 inhibition in the presence of Th17-polarizing cytokines, which could be attributed to blocking the inhibitory effect of p-STAT3 in Treg conversion. In a kinetic experiment of in vitro Th17 differentiation, we observed an increase in Foxp3$^+$ Tregs upon LMP7 inhibition only when STAT3-induced genes like IL-17 started to get stabilized, which was evident after 48 and 72 h of Th17-polarizing condition. These data point toward a role of LMP7 in regulating the level of p-STAT3, although this was not due to its increased proteasomal degradation. TGF-β drives the expression of both Foxp3 and RORγt (24, 26, 33), for which interaction limits the effect of RORγt (34, 35). However, IL-6–induced p-STAT3 counteracts the inhibitory effect of Foxp3 on RORγt, and the cell then proceeds to the Th17 lineage (36). There was a decrease in RORγt-positive cells and an increase in Foxp3-positive cells after LMP7-specific inhibition as evidenced by Foxp3/RORγt costaining of the Th17 culture. The effect of LMP7 inhibition on the phosphorylation of STAT3 might be responsible for the inability of the cells to overcome the inhibitory effect of Foxp3 on RORγt and thus the inability to convert to the Th17 lineage. The transcription factor Runx1 associates with RORγt to promote Th17 differentiation but also interacts with Foxp3 to inhibit Th17 differentiation, thus playing an important role in regulating Th17 differentiation (37). LMP7 could possibly be playing a role in modulating these interactions among Runx1, RORγt, and Foxp3, and it would be interesting to investigate if LMP7 inhibition affects Runx1.

We documented a lower phosphorylation of STAT1 upon LMP7 inhibition even after 1 h of Th1-polarizing conditions, thus pointing toward a direct role of LMP7 in driving Th1 differentiation. Apart

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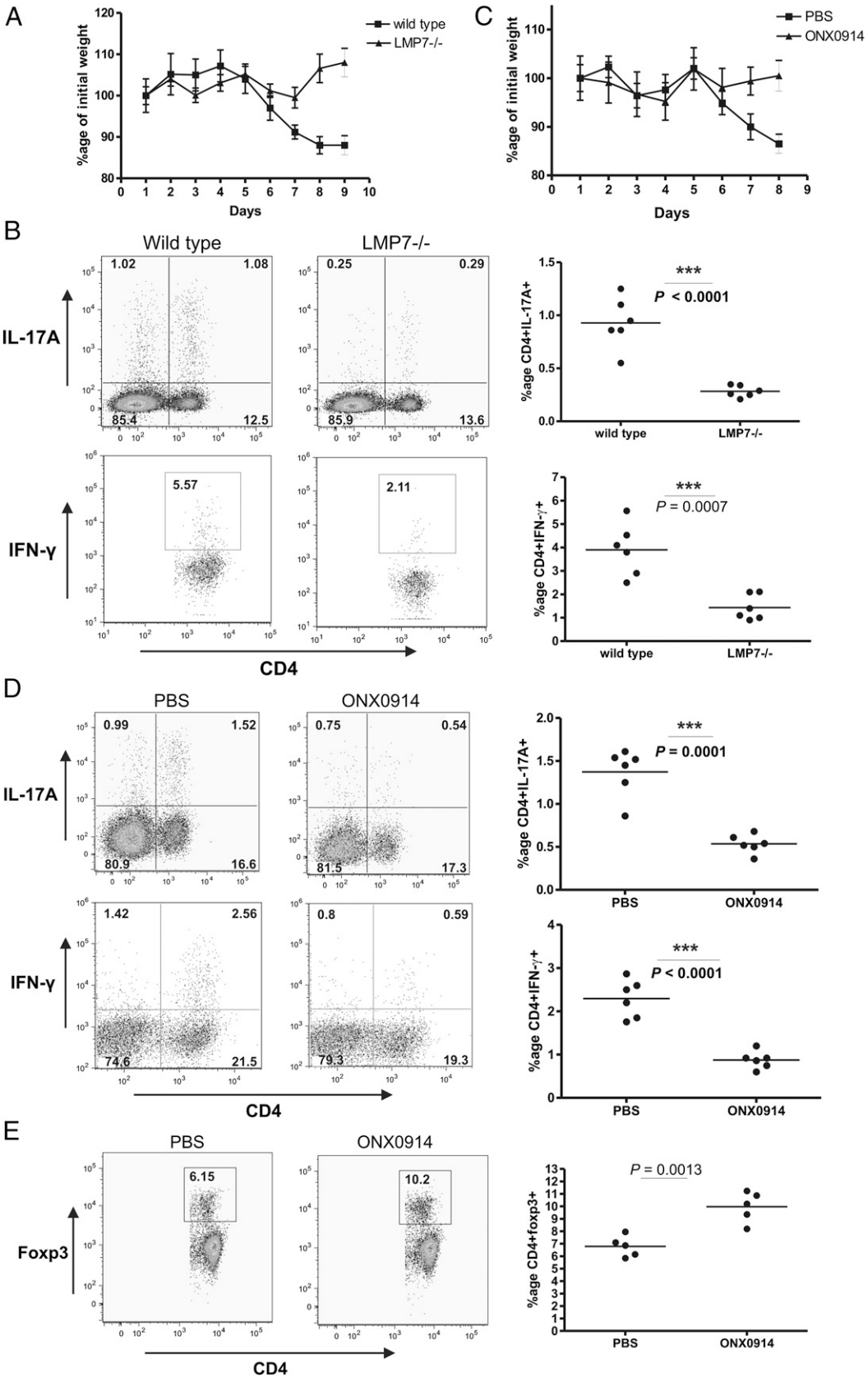

FIGURE 5. LMP7 inhibition blocks Th1 and Th17 and promotes Treg differentiation in vivo in DSS-induced colitis. The body weight was monitored in wt and LMP7$^{-/-}$ mice (**A**) and PBS- and ONX 0914-treated C57BL/6 mice (**C**) over a period of 10 d. (**B**) Flow cytometric analysis of lamina propria lymphocytes isolated from the colon of wt and LMP7$^{-/-}$ mice fed with DSS in drinking water for 5 d and sacrificed on day 9. Lamina propria lymphocytes were restimulated with anti-CD3/CD28 for 6 h (with brefeldin A for the last 5 h) and stained for IL-17 and IFN-γ. Each symbol in the graph represents an individual mouse ($n = 6$), and small horizontal lines indicate the mean. (**D**) Flow cytometric analysis of lamina propria lymphocytes isolated from the colon of C57BL/6 mice fed with DSS as in (B) and one group either untreated or treated with 10 mg/kg of ONX 0914 daily. Cells (*Figure legend continues*)

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from inhibiting Th1 and Th17 differentiation, we showed in this study that LMP7-specific inhibition upregulated Foxp3$^+$ Tregs even under Treg-polarizing conditions in vitro. Regulatory SMADs are important players in the TGF-β signaling pathway, and they are tightly regulated by SMAD ubiquitin regulatory factors (Smurfs) acting as E3 ligases (38). The increase in Foxp3$^+$ Tregs upon LMP7 inhibition was accompanied by an increased phosphorylation of SMAD2 and SMAD3. Because SMADs are mainly regulated through ubiquitination and degradation, it could be possible that LMP7 is directly involved in the degradation of these regulatory SMADs. Along with SMADs, it could also be speculated that LMP7 inhibition might be affecting the expression of Smurfs (38). We also showed that Tregs induced in the presence of ONX 0914 are functional and have suppressive capacity, but we could not see any difference in the suppressive capacity of Tregs induced in the presence or absence of ONX 0914.

CD4$^+$ T cells play an important role in the progression of inflammatory bowel disease, which in humans manifests in two forms: Crohn's disease characterized by Th1/Th17 phenotype and ulcerative colitis dominated by a Th2 cytokine profile (39). We used the DSS-induced colitis model, which, like Crohn's disease, is driven by Th1/Th17 cytokines in the chronic condition to confirm the phenotypes in vivo (40, 41). In an acute model of DSS-induced colitis, mice that were given DSS for 7 d exhibited an increase in IL-12 and IL-17, suggesting an induction of a Th1/Th17 profile (40). However in this study, protein and mRNA samples from whole colonic tissues and serum were analyzed and not Th cells. It was shown in IL-17A knockout mice that the disease severity was reduced in DSS-induced colitis. The involvement of Th17 cells in the pathogenesis of acute DSS-induced colitis is still a matter of controversial debate because recently it was shown that it is the Th1 cells and not Th17 cells that are pathogenic in acute DSS-induced colitis (42). Our study favors the involvement of Th17 cells in the pathogenesis of acute DSS-induced colitis because we saw a reduction in the Th17 cells in the colonic lamina propria by LMP7-specific inhibition or deficiency, which was accompanied by reduced disease severity. ONX 0914-treated mice showed less Th1 and Th17 cells in the colonic lamina propria in comparison with the untreated control mice. The inhibitory effect of ONX 0914 on the differentiation of these proinflammatory T cell lineages is likely to be responsible for the reduced disease progression. We also found an increase in Foxp3$^+$ Tregs in the mLN of ONX 0914-treated mice in comparison with untreated control, which could be a secondary effect of reduced Th17 differentiation and supports our in vitro data for a role of LMP7 in modulating Th17/Treg plasticity. LMP7-deficient animals also showed less Th1 and Th17 cells in comparison with wt mice in DSS-induced colitis.

The mucosal microflora also plays a crucial role in the models of mucosal inflammation by the stimulation of different effector CD4$^+$ T cell populations that causes inflammation. Recently, it has been shown that specific members of the commensal microbiota known as segmented filamentous bacteria, namely *Arthronitus*, are potent inducers of Th17 cells in the lamina propria of the mouse small intestine (43). Th17 cells are abundant in the intestinal lamina propria, and their differentiation is dependent on commensal microbes with specialized properties. Thus, manipulating the intestinal microflora, especially the segmented filamentous bacteria, could result in altering the course of Th17 cell-associated autoimmune diseases. To investigate whether the difference in Th17 cells seen in wt and LMP7$^{-/-}$ mice was due to the difference in their intestinal microbiota, we also performed the DSS experiment after cohousing both the strains of mice in a single cage for 2 to 3 wk so that they get enough time to share the same microflora to some extent. We saw reduced disease severity in LMP7$^{-/-}$ mice in comparison with wt mice and also saw less Th17 cells in the colonic lamina propria of LMP7$^{-/-}$ mice, which argues against the possibility of the difference in Th17 cells due to different intestinal microflora (Supplementary Fig. 4). In addition, we looked for the effect of LMP7-specific inhibition on Th1 and Th17 differentiation in a T cell transfer model of colitis. RAG2$^{-/-}$ mice reconstituted with naive T cells characterized by the CD4$^+$ CD45RB^{high} phenotype showed the presence of Th1 and Th17 cells in the spleen and mLN of the reconstituted mice 2 wk after transfer. Treatment of mice with ONX 0914 showed significantly less accumulation of Th1 and Th17 cells in comparison with the control group. Treatment with ONX 0914 would also inhibit the immunoproteasomes in other immune cells of RAG2$^{-/-}$ mice like monocytes and dendritic cells. This could lead to reduced production of Th17 polarizing cytokines like IL-6 and IL-23 from the dendritic cells, which could in turn affect the polarization of transferred naive CD4$^+$ T cells toward the Th17 lineage. To investigate this issue, we transferred naive T cells from wt and LMP7$^{-/-}$ mice into RAG2$^{-/-}$ mice and 2 wk posttransfer looked at the differentiation states of these transferred cells. LMP7$^{-/-}$ naive CD4$^+$ T cells showed reduced Th1 differentiation and enhanced Treg differentiation in comparison with wt mice upon transfer into RAG2$^{-/-}$ mice. So we could conclude that it is the immunoproteasome subunit LMP7-specific inhibition in the Th cells, which is responsible for these effects on different Th cell lineages. The effect on Th1 and Th17 differentiation was quite early (after 2 wk of transfer), pointing toward a role of LMP7 during the early stages of Th1 and Th17 differentiation. These in vivo data point toward a role of LMP7 in regulating different Th cell lineages in a proinflammatory environment that is responsible for disease progression in this colitis model. It could be possible that LMP7 is involved in the selective processing or degradation of so far uncharacterized regulatory proteins involved in determining different Th cell lineage fates, and we are currently pursuing this hypothesis. Along with Src homology region 2 domain-containing phosphatase and suppressor of cytokine signaling proteins, protein inhibitor of activated Stat (PIAS) proteins are another class of Stat regulators. PIAS1 specifically inhibits the DNA binding of activated Stat1 and PIAS3 was found to be specific for the inhibition of Stat3-mediated gene expression (44). The interaction of PIAS proteins with Stats requires the tyrosine phosphorylation of Stats. PIAS proteins are regulated by ubiquitination and subsequent proteasomal degradation (45). It could be possible that LMP7 is involved in the degradation of PIAS proteins and thus controls the activity of Stat1 and Stat3 and in turn the plasticity of Th17 and Tregs. It would be interesting to test if LMP7-specific inhibition leads to the inhibition of degradation of PIAS proteins, leading to its accumulation and causing these effects. Tyrosine phosphorylation on Stat3 is regulated by protein tyrosine kinases and protein tyrosine phosphatases. It could be possible that LMP7-specific inhibition might be stabilizing a phosphatase,

were restimulated as in (B). Each symbol in the graph represents an individual mouse (n = 6), and the horizontal line indicates the mean. (**E**) Flow cytometric analysis of mLN cells isolated from day 6 of DSS treatment of C57BL/6 mice with one group either untreated or treated with ONX 0914 as in (D). Each symbol in the graph represents an individual mouse (n = 6), and the horizontal line indicates the mean. Data are representative of at least three independent experiments.

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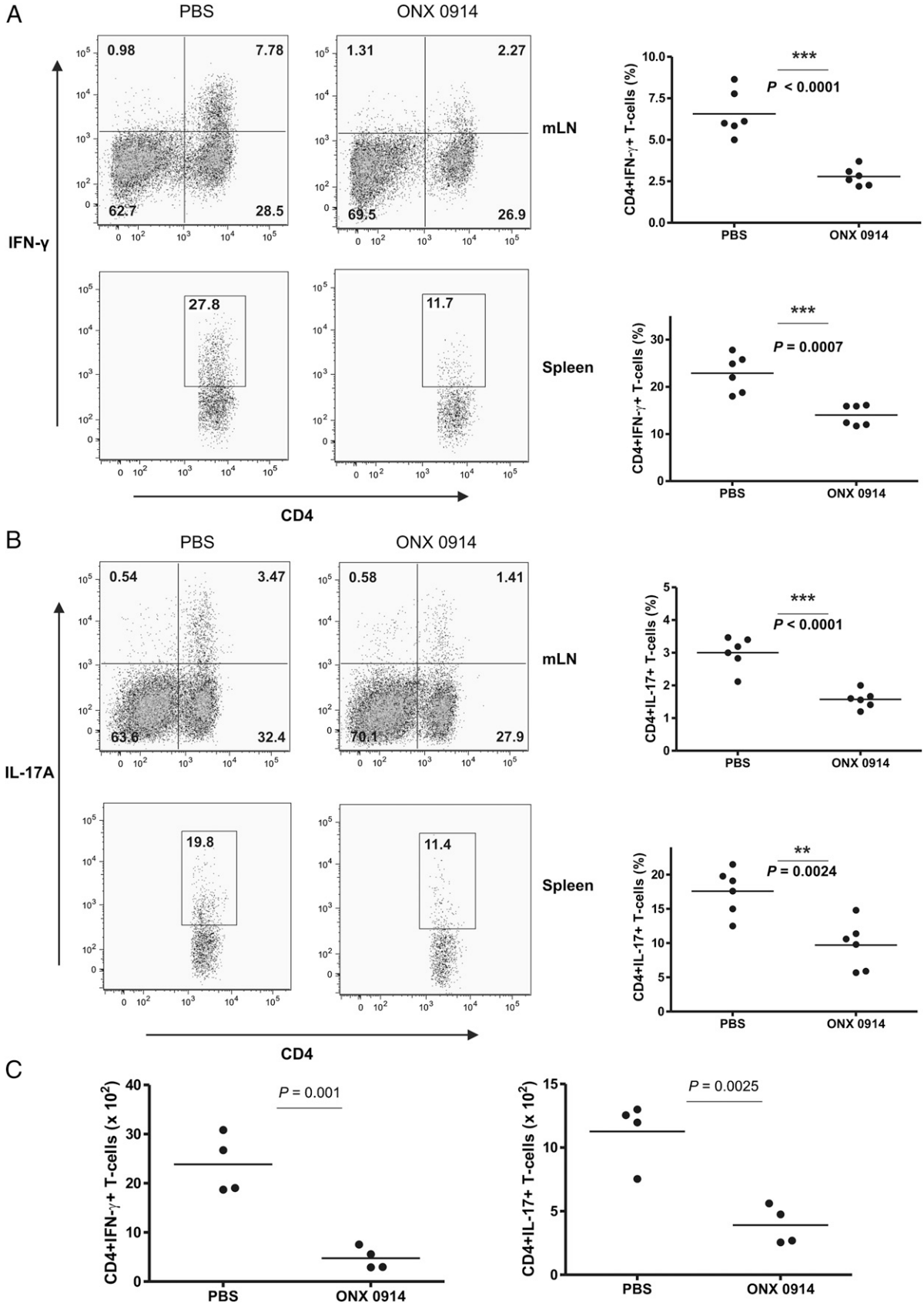

FIGURE 6. LMP7 inhibition leads to reduced Th1 and Th17 differentiation in vivo in a T cell-dependent transfer model of colitis. (**A** and **B**) Flow cytometric analysis of mLN and spleen from $RAG^{-/-}$ mice reconstituted with magnetically sorted $CD4^{+}CD62L^{+}$ T cells that were treated with either ONX 0914 or PBS control every alternate day. Two weeks after transfer, mice were sacrificed, and single-cell suspensions from mLN and spleen were restimulated with anti-CD3/CD28 for 5 h (with brefeldin A for the last 4 h) and intracellularly stained for IFN-γ (A) or IL-17A (B). Each symbol in the graph represents an individual mouse ($n = 6$), and small horizontal lines indicate the mean. (**C**) Absolute numbers of Th1 and Th17 cells in the mLN of mice in (A) and (B). Each symbol in the graph represents an individual mouse ($n = 6$), and small horizontal lines indicate the mean. Data are representative of at least two independent experiments.

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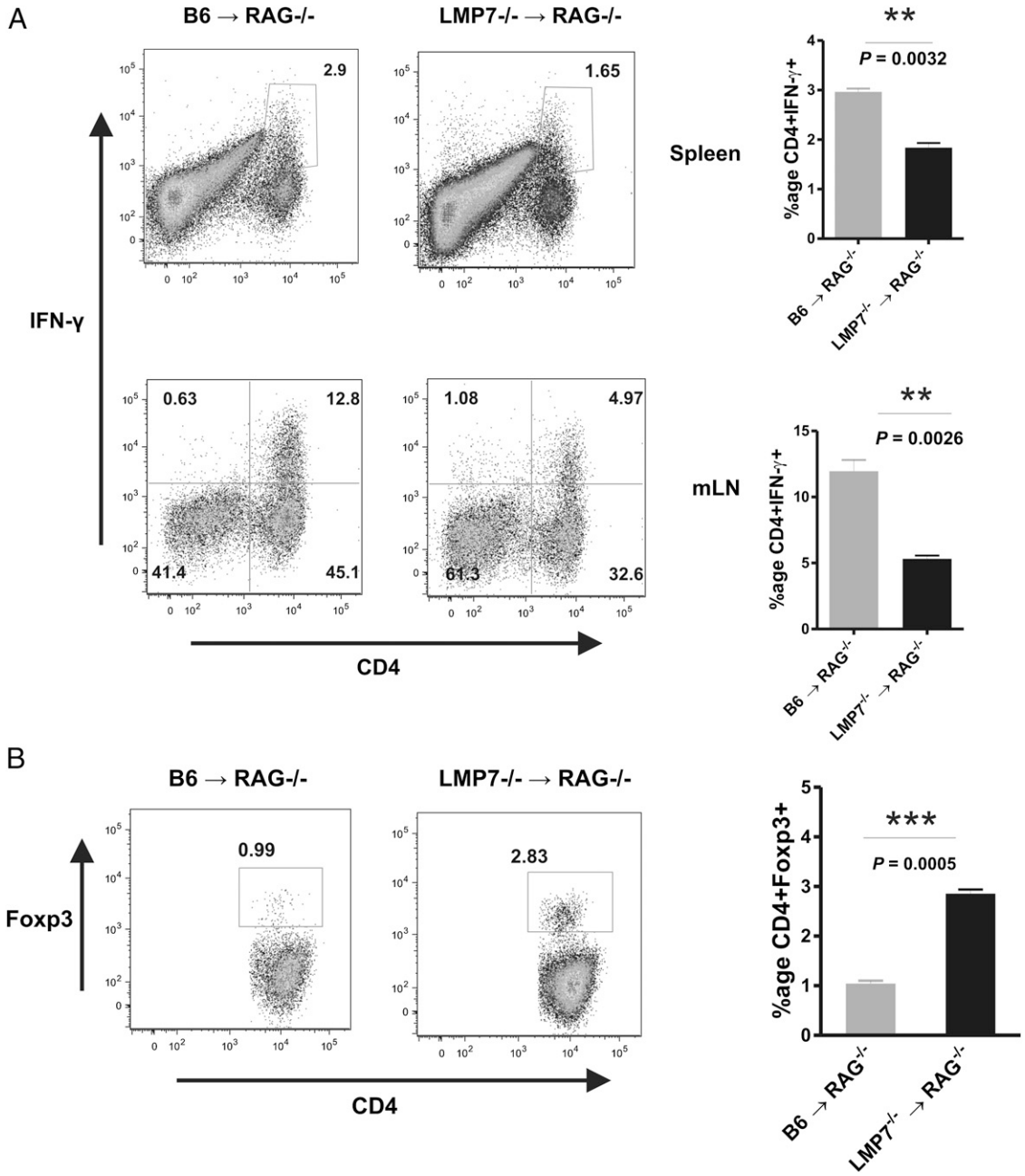

FIGURE 7. Immunoproteasome subunit LMP7-specific inhibition in Th cells is responsible for reduced Th1 differentiation and enhanced Treg differentiation in vivo. (**A** and **B**) Flow cytometric analysis of mLN and spleen cells from $RAG^{-/-}$ mice reconstituted with magnetically sorted $CD4^{+}CD62L^{+}$ T cells from wt or $LMP7^{-/-}$ mice. Two weeks after transfer, mice were sacrificed, and single-cell suspensions from mLN and spleen were restimulated with anti-CD3/CD28 for 5 h (with brefeldin A for the last 4 h) and intracellularly stained for IFN-γ (A) or Foxp3 (B).

which is specifically degraded by the LMP7 subunit of the immunoproteasome and in turn leading to the effects of reduced phosphorylation on Stats. A proteomic approach is needed to identify these putative phosphatases, which are specific substrates of the LMP7 subunit of immunoproteasome influencing different Th cell differentiation pathways.

Epigenetic modifications like posttranslational modifications of nucleosomal histones (e.g., methylation, acetylation, and phosphorylation) at the cytokine gene loci could be responsible for the plasticity of a particular Th lineage. Many of these histone-modifying enzymes are degraded via the ubiquitin-proteasome system (46), and it would be interesting to investigate whether LMP7 has any role in the degradation of these epigenetic modification enzymes, which could possibly explain the role of LMP7 in modulating the plasticity of these Th cell lineages. Foxp3 expression is reciprocally regulated by histone acetyltransferase p300 and the histone deacetylase SIRT1. Foxp3 is prevented from polyubiquitination and degradation when being hyperacetylated, leading to increased Foxp3 levels (47). It would be interesting to look whether LMP7 is specifically involved in the degradation of p300, as this histone-modifying enzyme is also degraded via the ubiquitin proteasome system. Also, it has been shown that inhibiting histone deacetylase SIRT1 leads to an increase in functional Foxp3 Tregs, and it could also be possible that a treatment with ONX 0914 is influencing SIRT1 activity, leading to upregulation of functional $Foxp3^{+}$ Tregs.

In conclusion, our data establish a previously undefined role of the immunoproteasome in governing Th cell differentiation. This

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novel immunomodulatory function of the immunoproteasome further qualifies this protease as an important target for the pharmacological treatment of autoimmune diseases.

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Disclosures

C.J.K. is an employee of and shareholder in Onyx Pharmaceuticals. The other authors have no financial conflicts of interest.

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