The Journal of Immunology

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J Immunol published online 25 January 2019
http://www.jimmunol.org/content/early/2019/01/24/jimmunol.1801425

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Published January 25, 2019, doi:10.4049/jimmunol.1801425

Abatacept Targets T Follicular Helper and Regulatory T Cells, Disrupting Molecular Pathways That Regulate Their Proliferation and Maintenance

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Abatacept is a CTLA-4-Ig fusion protein that binds to the costimulatory ligands CD80 and CD86 and blocks their interaction with the CD28 and CTLA-4 receptors expressed by T cells, therefore inhibiting T cell activation and function. Abatacept has shown clinical efficacy in treating some autoimmune diseases but has failed to show clinical benefit in other autoimmune conditions. The reasons for these disparate results are not clear and warrant further investigation of abatacept's mode of action. Longitudinal specimens from the Immune Tolerance Network's A Cooperative Clinical Study of Abatacept in Multiple Sclerosis trial were used to examine the effects of abatacept treatment on the frequency and transcriptional profile of specific T cell populations in peripheral blood. We found that the relative abundance of CD4$^+$ T follicular helper (Tfh) cells and regulatory T cells was selectively decreased in participants following abatacept treatment. Within both cell types, abatacept reduced the proportion of activated cells expressing CD38 and ICOS and was associated with decreased expression of genes that regulate cell-cycle and chromatin dynamics during cell proliferation, thereby linking changes in costimulatory signaling to impaired activation, proliferation, and decreased abundance. All cellular and molecular changes were reversed following termination of abatacept treatment. These data expand upon the mechanism of action of abatacept reported in other autoimmune diseases and identify new transcriptional targets of CD28-mediated costimulatory signaling in human regulatory T and Tfh cells, further informing on its potential use in diseases associated with dysregulated Tfh activity. ***The Journal of Immunology*****, 2019, 202: 000–000.**

The importance of CD28-mediated costimulation for T cell activation and function makes blockade of this pathway an attractive therapeutic target for autoimmune diseases in which dysregulated T cell activation plays a major role in pathogenesis. Indeed, by binding to CD80 and CD86 with very high affinity, CTLA4, a close CD28 homolog, acts as a natural antagonist of this pathway and is required for self tolerance (1, 2).

Abatacept is a fusion protein composed of the extracellular domain of CTLA4 and the Fc portion of Ig-G (CTLA4-Ig). It has proven efficacious for the treatment of rheumatoid arthritis, juvenile idiopathic arthritis, and psoriatic arthritis (3–7). Abatacept binding to CD80 and CD86 impairs CD28-mediated T cell costimulation and therefore inhibits the activation and priming of naive T cells and their subsequent expansion, differentiation, and function. Despite its theoretical advantages and proven therapeutic

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Received for publication October 26, 2018. Accepted for publication December 24, 2018.

This work was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Award UM1AI109565. Research reported in this publication was sponsored by the Immune Tolerance Network.

S.G. performed the experiments and assisted in the redaction of the manuscript. B.H. performed the bioinformatic analysis of RNAseq and generated the final figures. S.J.M. performed the cell sorting of T cell subsets and initial analysis of Treg cells. C.T. performed the cell sorting of T cell subsets. C.H. contributed to development and performance of flow cytometry assays. J.H.B. contributed to flow panel design and data interpretation. J.P. assisted with flow panel design, gating and quality control for data analysis. T.Q. collected data and assisted with data analysis and visualization. D.S., S.J.K., and L.D. contributed to concept development. L.A.T. and G.T.N. contributed to concept development and experimental design. K.M.H. contributed to experimental design, data interpretation and wrote the manuscript. E.B. and D.J.C. designed the experiments, supervised the analysis of the data, and wrote the manuscript. All authors made contributions to the final manuscript prior to submission.

The sequence data presented in this article have been submitted to the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE121827) under accession number GSE121827.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ABA, abatacept; ACCLAIM, A Cooperative Clinical Study of Abatacept in Multiple Sclerosis; DE, differentially expressed; GO, gene ontology; MS, multiple sclerosis; PL, placebo; RRMS, relapsing-remitting multiple sclerosis; Tfh, T follicular helper; Tfr, T follicular regulatory; Tph, T peripheral helper; Treg, regulatory T; TSDR, Treg-specific demethylation region; v, visit.

efficacy in some immune-mediated disease, CTLA4-Ig failed to show clinical benefit for the treatment of recent-onset type one diabetes (8), lupus nephritis (9), and relapsing-remitting multiple sclerosis (RRMS) (10). The reasons for these divergent results are currently unclear and warrant further interrogation of abatacept's mode of action.

$CD4^+$ T cell responses can be assigned into distinct categories based on the differential production of key effector cytokines such as IFN-γ (Th1), IL-4 (Th2), IL-17 (Th17), and IL-21 (T follicular helper [Tfh] and T peripheral helper [Tph]). Although CD28-mediated costimulation is thought to be a common event in the priming and activation of each of these effector T cell populations, the role of CD28 in the maintenance and function of ongoing effector T cell responses is less clear. CD28 and CTLA-4 are also implicated in the development, function, and homeostasis of $CD4^+Foxp3^+$ regulatory T (Treg) cells, raising questions about how CD28 blockade might impact the balance between effector and regulatory cell function.

The ACCLAIM (A Cooperative Clinical Study of Abatacept in MS) trial was a phase II placebo-controlled study of abatacept (CTLA4-Ig) in subjects with RRMS conducted by the Immune Tolerance Network from 2010 to 2016 (10). Although no clinical benefit was observed from treatment with abatacept, cryopreserved peripheral blood specimens from this placebo-controlled, crossover design trial provided a unique opportunity to study the effects of abatacept treatment on immune cell populations in the absence of background immunosuppression. In this study, we used a combination of flow cytometry, epigenetic, and RNA sequencing technologies to show that abatacept therapy significantly and selectively impacts the maintenance of circulating Treg and Tfh cells.

Materials and Methods

Study population

Sixty-five participants with active RRMS were enrolled in the phase II, double-blind, placebo-controlled trial ACCLAIM. The trial was registered as NCT01116427, received institutional review or ethics board approval at each site, and was conducted in accordance with the International Conference on Harmonization Guidelines for Good Clinical Practice and the Declaration of Helsinki.

Flow cytometry

PBMCs of the ACCLAIM cohort were isolated and stored frozen until use. Vials of cryopreserved PBMC were thawed at 37˚C in a water bath before immediately being diluted drop-wise into warm RPMI 1640 containing 10% FCS (Lonza). Surface markers were stained using Ab mixtures prior to fixation and permeabilization with Foxp3/Transcription Factor Staining Buffer Kit according to the manufacturer instructions (Tonbo Biosciences). Markers and cell types that were analyzed are listed on Supplemental Table I. Eighteen-parameter cytometry was performed on an LSR III Fortessa flow cytometer (BD Biosciences) with FACSDiva software and analyzed with FlowJo software version 9.9 (Tree Star, Ashland, OR). A panel defining Treg and Tfh cells included mAbs directed against the following markers: CCR7 (15050), PD1 (EH12.2H7), CCR6 (11A9), CXCR5 (RF8B2), CD28 (CD28.2), CD57 (NK-1), CD38 (HIT2), CD45RO (UCHL1), CXCR3 (G025H7), CD8 (RPA-T8), Foxp3 (236A/E7), CD27 (0.323), CD45RA (HI100), CD127 (HIL-7R-M21), CD3 (SK7), ICOS (C398.4A), CD4 (SK3), plus a LIVE/DEAD stain. A panel defining B cell subsets and plasmablasts included mAbs directed against the following markers: CD19, CD24, IgD, IgM, CD27, CD38, plus a LIVE/DEAD stain. Abs and reagents were purchased from BioLegend and Becton Dickinson. All longitudinal samples from the same subject were run on the same day. Samples from two to four subjects from each group (abatacept [ABA] → placebo [PL] and PL → ABA) were run the same day along with a technical standard to ensure repeatability. Gating strategies for Tfh and Treg cells are detailed in Supplemental Fig. 1C). An average of 300,000 live lymphocytes were collected, and samples with <50 events for evaluated populations at week 0 were excluded from longitudinal analysis.

For flow cytometry, differences between Per Protocol treatment groups at weeks 4, 16, and 24 were compared in the core phase using a linear mixed model with baseline adjustment. The impact of abatacept in the extension phase at weeks 32, 44, and 52 was compared with week 24 values within the Per Protocol group that received abatacept after crossover using a linear mixed model. The p values <0.05 were considered significant. Statistical analyses were performed with SAS version 9.4 (SAS Institute, Cary, NC) and R version 3.4.4.

Epigenetic Treg analysis

Cryopreserved PBMC were provided to Epiontis ID for batched DNA isolation and real-time–based quantification of the Treg-specific demethylation region (TSDR) of FOXP3 (11). GAPDH demethylation was used to determine the frequency of natural Treg cells with demethylated TSDR as the percentage of total PBMC with epigenetically active GAPDH (Epiontis ID).

RNA sequencing

Populations were sorted from 30 different donors from blood draws at three different visits (v0, v7, and v15), corresponding to weeks 0, 24, and 52. Five hundred cells of each subset were sorted directly into SMART-seq v4 (Takara) lysis buffer to release RNA, and RT-PCR was then used to generate cDNA. Sequencing libraries were constructed using a modified protocol of the Nextera XT DNA sample preparation kit (Illumina). Libraries were pooled and quantified by Qubit Fluorometer (Life Technologies). Dual-index, single-read sequencing of the pooled libraries was carried out on a HiSeq 2500 sequencer with 58-base reads, using HiSeq v4 Cluster and Sequencing by Synthesis kits with a target depth of 5 million reads per sample. Basecalls were processed to FASTQs on BaseSpace (Illumina), and a base call quality trimming step was applied to remove low-confidence base calls from the ends of reads. The FASTQs were aligned to the human reference genome, using TopHat v.1.4.1, and gene counts were generated using htseq-count. Quality control and metrics analysis were performed, using the Picard family of tools (v1.134).

Samples with a percent alignment <80% and median coefficient of variation for coverage >0.7 were considered bad quality and were removed from downstream analysis. Three samples (one from each population) failed quality control leaving us with 222 samples from 30 different donors that were used in the data analyses. To determine the effect of abatacept treatment on the transcriptome, RNA sequencing before and after abatacept treatment was compared, combining samples from the core and the extension phase.

Differentially expressed (DE) genes were assessed by fitting a linear model with timepoint (before, after, and at withdrawal), median coefficient of variation coverage, and donor sex as covariates as well as considering donor effects through a random factor.

Statistical analysis was performed in R (v 3.4.4) using the Bioconductor package limma (v 3.34.9). Genes with an adjusted p value <0.05 were considered significant.

Data and materials availability. Data sets for these analyses are accessible through TrialShare, a public Web site managed by the Immune Tolerance Network (https://www.itntrialshare.org/ACCLAIM_MOA.url). The RNA sequencing data have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus and are accessible through Gene Expression Omnibus Series accession number GSE121827 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE121827).

Results

ACCLAIM study design

Enrolled participants in ACCLAIM were randomly assigned to either i.v. abatacept or placebo treatment (Supplemental Fig. 1A). Abatacept was administered at weeks 0, 2, and 4 and then every 4 wk through week 24, designated the core phase. During the extension phase (weeks 28–52), participants in the core phase placebo group received treatment with abatacept at weeks 28, 30, and 32 and then every 4 wk through week 52 (PL→ABA). Participants in the core phase abatacept group received treatment with placebo according to the same treatment schedule (ABA→PL) (10).

Abatacept decreases the frequency of $CD45RO^+$ memory Treg cells

To determine the effect of abatacept treatment on T cells, we performed extensive flow cytometry analysis of PBMC from subjects with RRMS in the ACCLAIM trial, focusing on cell populations likely to be direct targets of abatacept-mediated costimulatory

blockade (Supplemental Table I). Of these, the only populations whose abundance was significantly impacted by abatacept treatment were Treg cells and Tfh cells (data not shown). Abatacept treatment did not significantly impact the overall proportions of CD45RA$^+$ naive or CD45RO$^+$ memory Foxp3$^-$CD127$^+$ CD4$^+$ non-Treg cells (Supplemental Fig. 1B). Therefore, we focused our subsequent studies on a more detailed analysis of Treg and Tfh populations. Because Treg cells are profoundly modulated by costimulatory signals, we first analyzed the percentage of Foxp3$^+$CD127$^{-/lo}$ Treg among total CD4$^+$ T cells in peripheral blood (Supplemental Fig. 1C), and observed a reduction in the relative frequency of total Treg cells among CD4$^+$ T cells as early as 4 wk after abatacept treatment compared with placebo (Fig. 1A). This decrease was sustained through the active treatment phase and was significantly different from the placebo group. When abatacept was discontinued at week 24, the percentages of circulating Treg cells returned to pretreatment levels. Conversely, percentages of Treg cells were relatively stable in the placebo group throughout the initial 24 wk and then declined after these subjects received abatacept treatment (Fig. 1A).

Upon activation, human effector T cells can transiently upregulate Foxp3 but can be distinguished from Foxp3$^+$ Treg cells by analyzing the methylation status of the TSDR in the FOXP3 gene (11). Selective demethylation of the TSDR in the FOXP3 gene leads to stable FOXP3 expression and defines Treg cells (12). The percentage of cells with demethylated TSDR decreased among PBMC of abatacept-treated patients and returned to pretherapy levels when participants were given placebo (week 52) (Fig. 1B). Participants treated with placebo in the initial 24 wk also exhibited a decline in cells with demethylated TSDR after receiving abatacept treatment (Fig. 1B). Thus, loss of Foxp3$^+$CD127$^{-/lo}$ CD4$^+$ T cells upon abatacept treatment is not due to general suppression of T cell activation but rather represents selective loss of bona fide Treg cells with demethylated TSDR.

The expression of CD45RA and CD45RO can differentiate between naive and memory Treg cells, which differ in their functions and suppression activity (13). Therefore, we next analyzed whether abatacept was modulating both populations of Treg cells equally. Abatacept had no effect on the percentage of CD45RA$^+$ naive Treg cells (Fig. 1C). In contrast, abatacept profoundly diminished frequencies of CD45RO$^+$ memory Treg cells (Fig. 1D), suggesting that costimulatory blockade specifically targets the subset of T regulatory cells previously activated by Ag.

Abatacept reduces frequencies of circulating Tfh cells and plasmablasts

Tfh cells efficiently provide B cell help, facilitate the formation of germinal centers, and stimulate the production of high-affinity class-switched Abs (14, 15). We analyzed the percentage of CXCR5$^+$PD1$^+$CD45RO$^+$ cells, which define Tfh cells among circulating CD4$^+$ T cells (Supplemental Fig. 1B). Participants treated with abatacept had a progressive decline in the percentage of Tfh cells (Fig. 2A, 2B). In contrast to the early decrease in blood Treg cell abundance observed at week 4 (Fig. 1A), the decline in Tfh cells was observed later, at week 16 (Fig. 2B). Percentages of Tfh cells in abatacept-treated participants returned to pretherapy levels ~20 wk (week 44) after abatacept cessation. Consistent with these data, the percentage of Tfh cells in the placebo group significantly decreased 16 wk (week 44) after initiation of abatacept treatment at week 28. Both PD1$^-$CXCR5$^+$ and PD1$^+$CXCR5$^-$ CD45RO$^+$CD4$^+$ T cells have been ascribed effector function similar to Tfh cells in other studies (16, 17). However, in contrast to blood Tfh cells, the relative frequencies of PD1$^-$CXCR5$^+$ and PD1$^+$CXCR5$^-$ cells were not significantly modulated following abatacept treatment (Fig. 2C, 2D). Thus, abatacept has a selective inhibitory effect on the frequencies of circulating Tfh cells in this study cohort.

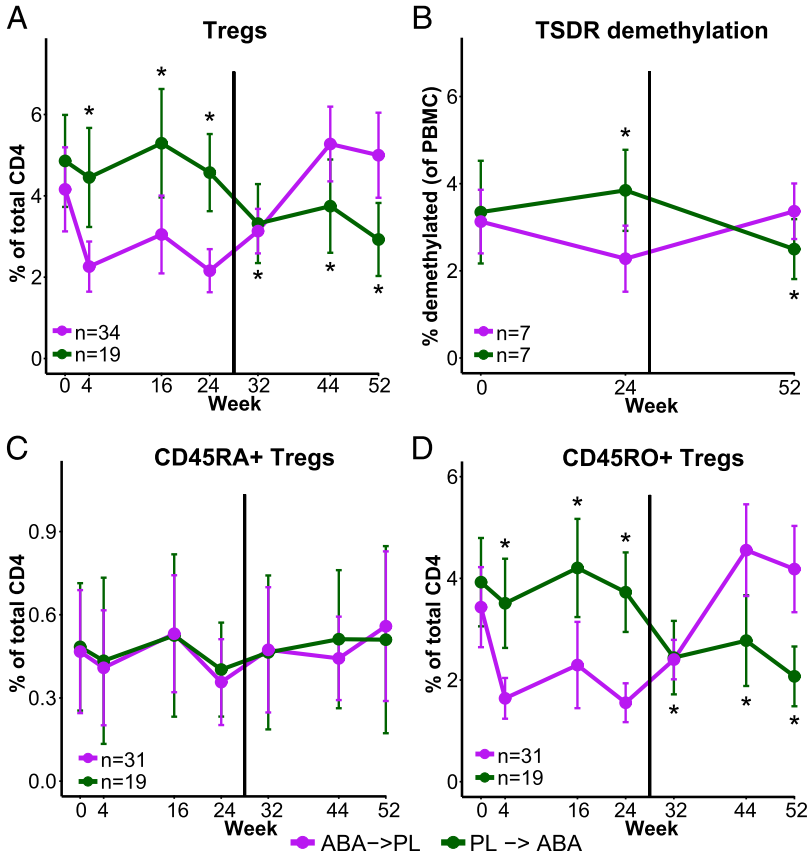

FIGURE 1. Abatacept treatment decreases the frequency of CD45RO$^+$ Treg cells. PBMC from participants treated with abatacept or placebo were labeled with mAb to identify memory and naive Treg cells. Data show the mean frequencies of Treg cells (CD4$^+$CD127$^-$Foxp3$^+$) (**A**), naive Treg cells (CD4$^+$CD127$^-$Foxp3$^+$CD45RA$^+$) (**C**), and memory Treg cells (CD4$^+$CD127$^-$Foxp3$^+$CD45RO$^+$) (**D**), expressed as a frequency of total CD4$^+$ T cells. (**B**) Quantitative PCR of TSDR and CD3 demethylation was performed on DNA from PBMC. Data shown are the mean frequency of TSDR$^+$ cells as a percentage of PBMC from a subgroup of participants in the two treatment arms. The purple line represents the group of participants receiving abatacept followed by placebo (ABA→PL), and the green line represents the group of participants receiving placebo followed by abatacept (PL→ABA). The vertical line indicates the timing of treatment crossover. Participants with <50 events for evaluated populations at week 0 were excluded from longitudinal analysis. Differences between Per Protocol treatment groups at weeks 4, 16, and 24 were compared in the core phase using a linear mixed model with baseline adjustment. The impact of abatacept at weeks 32, 44, and 52 was compared with week 24 values within the Per Protocol group that received abatacept after crossover (PL→ABA) using a linear mixed model. Error bars display the 95% confidence intervals. Data for this figure available at: https://www.itntrialshare.org/ACCLAIM_MOA_fig1.url. *$p < 0.05$.

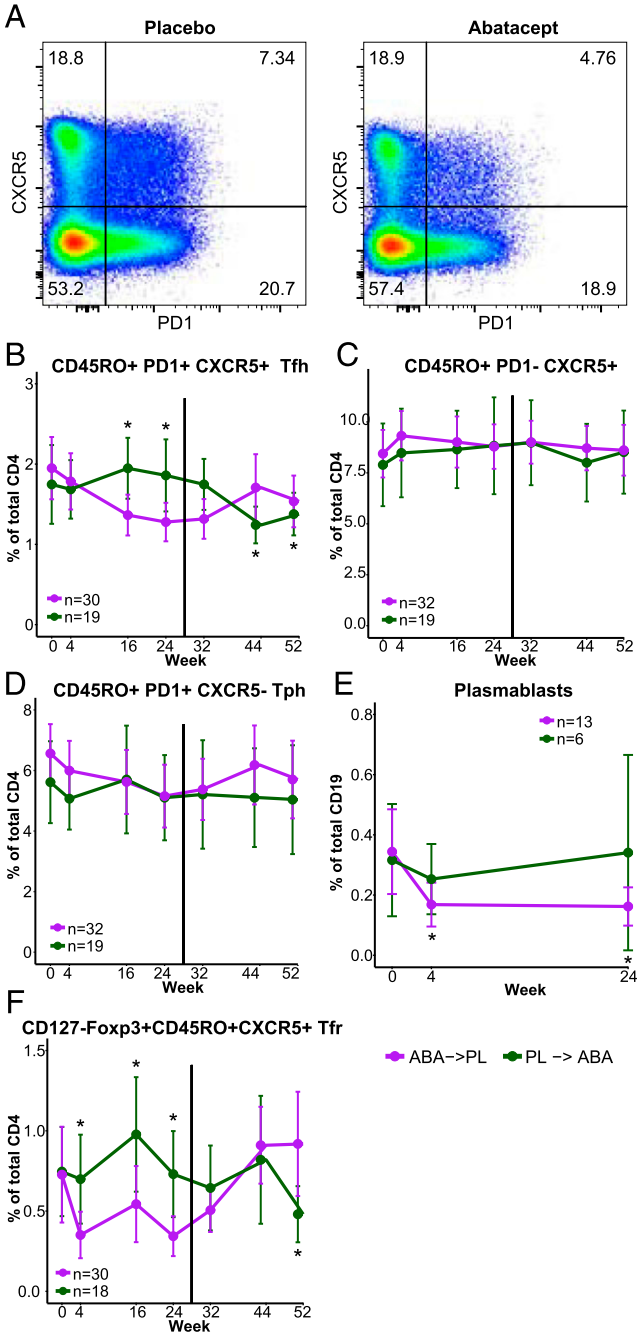

FIGURE 2. Abatacept reduces the frequency of circulating Tfh cells. (**A**–**D**) PBMC from participants treated with abatacept or placebo were thawed and labeled with mAb to identify Tfh cells. (A) Representative staining of CXCR5 and PD1 in memory CD4 T cells ($CD4^+CD127^+Foxp3^-CD45RO^+$) of participants treated with abatacept (right) or placebo (left). Data show the mean frequency of $CD45RO^+PD1^+CXCR5^+$ Tfh cells (B), $CD45RO^+PD1^-CXCR5^+$ cells (C), and $CD45RO^+PD1^+CXCR5^-$ Tph cells (D) in participants expressed as percentage of total $CD4^+$ T cells. In a subgroup of participants, PBMC were stained with CD19, IgD, CD27, CD24, and CD38 to identify plasmablasts. Data shown are the mean frequency of plasmablasts ($CD19^+CD24^{lo}CD38^{++}IgD^-CD27^+$) as a percentage of total $CD19^+$ B cells (**E**). To identify Tfr cells, PBMC were stained with CXCR5 in memory Treg cells ($CD4^+CD127^-Foxp3^+CD45RO^+$). Data show the mean frequency of Tfr cells as percentage of total $CD4^+$ T cells (**F**). The purple line represents the group of participants receiving abatacept followed by placebo (ABA→PL) and the green line represents the group of participants receiving placebo followed by abatacept (PL→ABA). The vertical line indicates the timing of treatment crossover. Participants with <50 events for evaluated populations at week 0 were excluded from longitudinal analysis. Differences between Per Protocol treatment groups at weeks 4, 16, and 24 were compared in the core phase using a linear mixed model with baseline adjustment. The impact of abatacept at weeks 32, 44, and 52 was compared with week 24 values within the Per Protocol group that received abatacept after crossover (PL→ABA) using a linear mixed model. Error bars display the 95% confidence intervals. Data for this figure available at: https://www.itntrialshare.org/ACCLAIM_MOA_fig2.url. *$p < 0.05$.

B cells require Tfh cells to progress through the germinal center stage and differentiate into class-switched, Ab-producing cells. For this reason, the frequency of circulating plasmablasts strongly correlates with the proportion of circulating Tfh cells (18). Therefore, we next quantified the percentage of circulating plasmablasts in a subgroup of study participants ($n = 19$) treated with abatacept ($n = 6$) or placebo ($n = 13$) in the initial 24 wk. The participants treated with placebo had relatively constant percentages of plasmablasts between week 0 and week 24, whereas a decrease in the relative frequency of plasmablasts was observed during abatacept treatment (Fig. 2E). T follicular regulatory (Tfr) cells are a recently defined specialized subset of effector Treg cells that suppress B cell responses and inhibit Ab production (19–22). Therefore, we next analyzed whether the decrease in plasmablasts observed upon abatacept treatment could be the result of an increase in Tfr cells. However, consistent with the impact of abatacept on global $CD45RO^+$ Treg cells (Fig. 1D), percentages of $CD127^{-/lo}Foxp3^+CD45RO^+CXCR5^+$ Tph cells decreased by week 4 and returned to initial levels following abatacept discontinuation (Fig. 2F). Thus, the reduction in the percentages of circulating plasmablasts observed during abatacept treatment cannot be explained by an increase in Tfr cell abundance but instead is likely related to the decrease in Tfh cell frequencies.

Abatacept decreases activated Treg and Tfh cells in peripheral blood

The dynamics of Treg and Tfh cells during abatacept treatment prompted us to address whether abatacept treatment also affected their activation status. Because recently activated Tfh cells could be identified by the expression of CD38 and ICOS (23, 24), these activation markers were assessed on Treg and Tfh cells. Among participants treated with abatacept, we observed at week 4 a profound decrease in the percentage of $CD45RO^+$ Treg cells expressing $CD38^+$ (Fig. 3A). However, by week 16 and 24, percentages of $CD45RO^+$ Treg cells expressing CD38 had returned to levels similar to those observed at week 0 (Fig. 3A). The percentage of $CD45RO^+$ Treg cells that expressed ICOS followed the same pattern and kinetics of transient downmodulation at week 4 with gradual return to normal levels by the end of abatacept treatment (Fig. 3B). Abatacept therapy was associated with durable decreases in the percentage of $CD38^+$ (Fig. 3C) and $ICOS^+$ Tfh cells in peripheral blood (Fig. 3D).

Transcriptome analysis defines a reversible signature of abatacept treatment in Treg and Tfh cells

Because abatacept selectively modulated the frequency and activation of Treg and Tfh cells, we next determined if abatacept treatment also altered the core transcriptional profiles of these lineages by comparing transcriptional signatures of bulk-sorted Treg and Tfh cells from cryopreserved PBMC at baseline, 24 wk after initiation of abatacept treatment, and after abatacept withdrawal when patients switched from abatacept treatment to placebo during the extension phase of the trial (Supplemental Fig. 1A). As a control, we also examined $CD4^+CD45RO^+PD1^+CXCR5^-$ Tph cells, whose abundance was not changed during treatment (Fig. 2D). Consistent with the

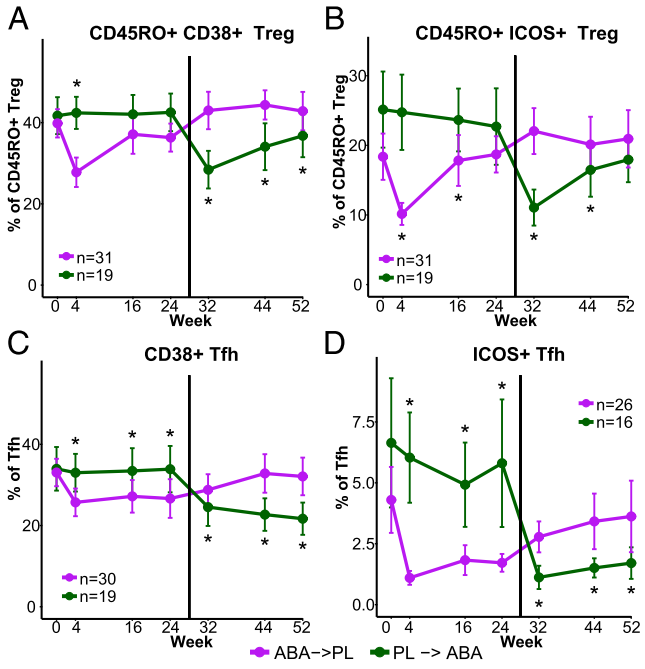

FIGURE 3. Abatacept limits the activation levels of Tfh and Treg cells. (**A**–**D**) PBMC from participants treated with abatacept or placebo were evaluated for the activation status of Treg and Tfh cells. Data show the frequency of $CD38^+$ or $ICOS^+$ cells as percentage of memory Treg cells (A and B) or as percentage of Tfh cells (C and D). The purple line represents the group of participants receiving abatacept followed by placebo (ABA→PL) and the green line represents the group of participants receiving placebo followed by abatacept (PL→ABA). The vertical line indicates the timing of treatment crossover. Participants with <50 events for evaluated populations at week 0 were excluded from longitudinal analysis. Differences between Per Protocol treatment groups at weeks 4, 16, and 24 were compared in the core phase using a linear mixed model with baseline adjustment. The impact of abatacept at weeks 32, 44, and 52 was compared with week 24 values within the Per Protocol group that received abatacept after crossover (PL→ABA) using a linear mixed model. Error bars display the 95% confidence intervals. Data for this figure available at: https://www.itntrialshare.org/ACCLAIM_MOA_fig3.url. *$p < 0.05$.

results of ICOS protein expression obtained by flow cytometry (Fig. 3C, 3D), abatacept therapy was associated with a decrease in *ICOS* gene expression at week 24 in Tfh cells (Supplemental Fig. 2) but not in Treg cells (data not shown). However, analysis of the other core gene signatures of Tfh and Treg cells showed they were not significantly altered by abatacept treatment. Therefore, although costimulatory blockade impacts the abundance and activation status of Tfh and Treg cells, it does not alter their stability and identity (Fig. 4A).

Further analysis of transcriptome data identified 96 and 64 genes that were differentially expressed (DE) (adjusted p value <0.05) after 24 wk of abatacept treatment in Tfh and Treg, respectively (Fig. 4B). By contrast, abatacept treatment had a minimal impact on gene expression in $CD4^+CD45RO^+$ $PD1^+CXCR5^-$ Tph cells, with only three DE genes (data not shown). Plotting the position of DE genes in Tfh onto a ranked list of gene expression change in Treg cells showed that the overall response to the treatment was similar in the two subsets, as the set of genes upregulated by abatacept in blood Tfh cells was also upregulated in Treg cells, and the set of genes downregulated in Tfh cells was also downregulated in Treg cells (Fig. 4C). Hierarchical clustering demonstrated that changes in gene expression were transient, and reversed upon discontinuation of abatacept (Fig. 4D, 4E).

Functional analysis of DE genes in Tfh was performed using gene ontology (GO) term enrichment analysis. This analysis revealed nine significantly enriched GO categories, which were related to processes involved in cell division and proliferation (Fig. 5A). DE genes in the enriched GO categories showed a highly significant correlation in Tfh and Treg cells, further highlighting the similarity in the response of these two populations to abatacept (Fig. 5B). Notably, 6 of the 10 genes that were DE in both Tfh and Treg cells (*HELLS, KNL1, SMC4, CENPE, NUSAP1, TOP2A*) were related to cell division, and their expression was downregulated in both subsets during abatacept treatment and normalized to pretreatment levels following abatacept withdrawal (Fig. 5C, 5D).

Discussion

The fundamental discovery that costimulatory and coinhibitory receptors profoundly influence T cell activation and differentiation has opened the door to therapeutic manipulation of these pathways to treat a variety of immune-mediated diseases. In this study, we investigated immunological changes in patients with RRMS that participated in the ACCLAIM trial to elucidate the cellular and molecular targets of costimulatory blockade with CTLA4-Ig. Consistent with previous studies (25, 26), we observed significant reductions in the relative frequencies of $CD45RO^+$ Treg and Tfh cells in circulating $CD4^+$ T cells of RRMS participants treated with abatacept. This was paralleled by a decline in the relative abundance of circulating plasmablasts. In addition, abatacept reduced the relative frequencies of circulating $CD45RO^+$ Treg and Tfh cells expressing the activation markers CD38 and ICOS.

Transcriptional analysis of circulating Treg and Tfh cells bulk sorted from subjects pre– to post–abatacept treatment paralleled drug-induced changes in activation status by flow cytometry. Importantly, these studies also identified a core set of genes involved in cell division and chromatin dynamics that was selectively modulated by abatacept in Treg and Tfh cells and not $CD4^+CD45RO^+$ $PD1^+CXCR5^-$ Tph cells. This result is consistent with a role for CD28-mediated costimulation in directly promoting cell-cycle progression in activated T cells (27) and likely underlies at least in part the decline in Tfh and Treg cell frequencies observed with abatacept treatment. A recent study indicated that CD28-mediated costimulation promotes Treg cell recruitment from the blood into peripheral tissues (28). Thus, it is unlikely that the reduced frequencies of Tfh and Treg cells in the circulation during abatacept treatment is due to their migration and sequestration in other organs. The cellular and molecular responses associated with abatacept therapy in both Treg and Tfh cells reversed upon discontinuation of treatment, indicating that costimulatory blockade does not result in permanent reprograming of these cells. Instead, abatacept therapy may restrict their proliferation in response to Ag–receptor engagement. These data expand upon the mechanism of action of abatacept reported in other autoimmune diseases and identify new transcriptional targets of CD28-mediated costimulatory signaling in human Treg and Tfh cells.

Many studies have examined the role of specific costimulatory and coinhibitory receptors in the development and function of Tfh and Treg cells. Blockade of CD28 limited germinal center formation and reduced numbers of Tfh cells (29–34) in mice, implicating CD28 signaling as a key step in Tfh cell development. Conversely, deficiencies in either CTLA4 itself (22, 35) or in the LPS-responsive beige-like anchor protein (LRBA), which promotes the intracellular transport of CTLA4 toward the cell membrane, give rise to autoimmune disease, with dramatically increased frequencies of circulating Tfh cells, a phenotype reversed by CTLA4-Ig therapy (36–38). Thus, CD28 and CTLA4 signaling have opposing effects on Tfh generation. Because our studies show an overall decrease in the

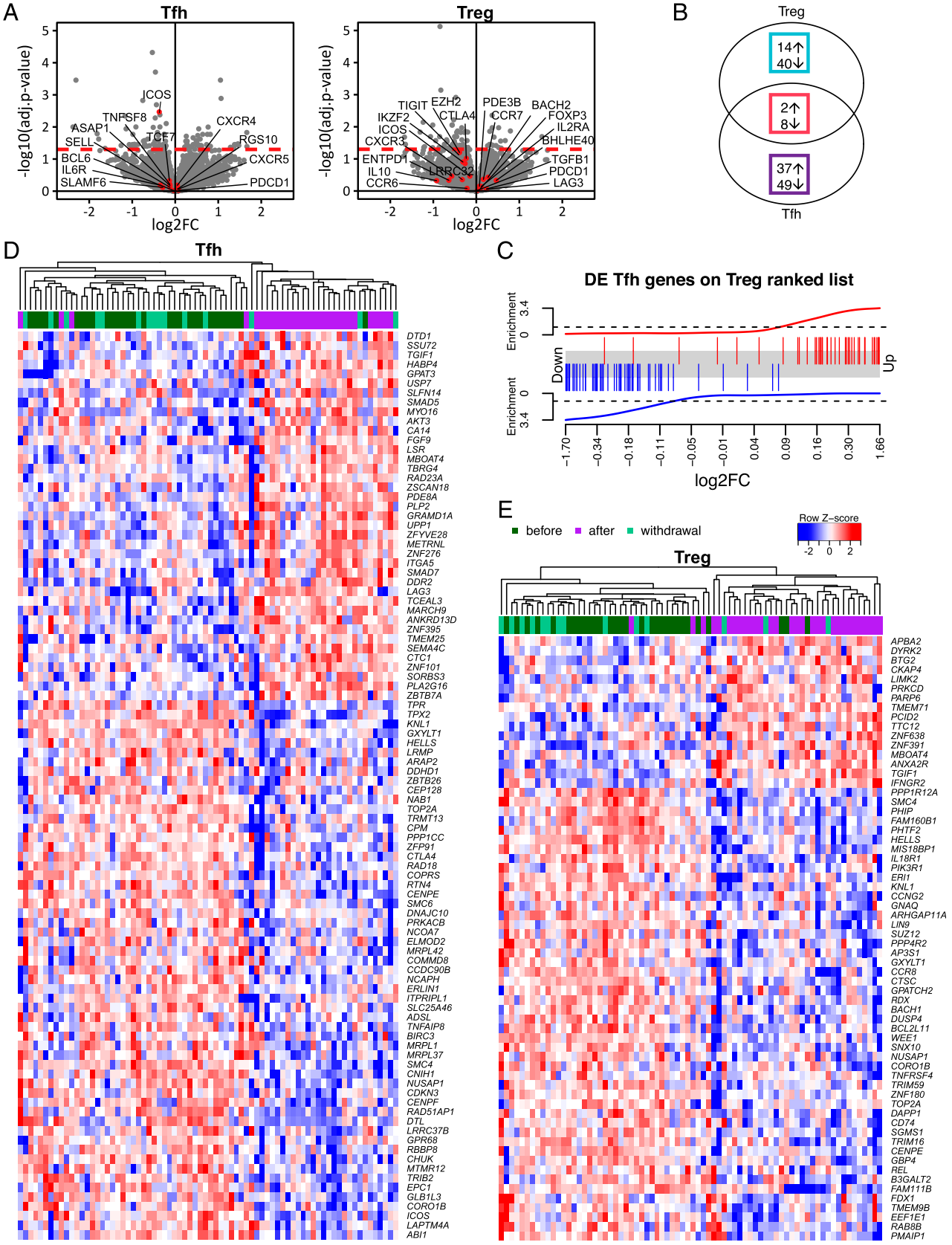

FIGURE 4. Abatacept impacts Tfh and Treg cell gene expression without altering core lineage signatures. (**A**) Volcano plots show the log2FC of all genes before versus after abatacept treatment in Tfh and Treg cells and highlight the location of core lineage genes. (**B**) Number and overlap of DE genes in both populations upon abatacept treatment. (**C**) Barcodeplot showing ranked list log2FC in expression in Treg upon treatment. Position of up- and downregulated genes from Tfh cells are indicated by colored bars. Enrichment of the genesets is shown by the lines above and below the plot. (**D** and **E**) Heatmaps show z-transformed log expression values of DE genes in the indicated population. The dendrogram on top of the heatmap represents hierarchical clustering by Euclidean distance on log expression values. Data for this figure available at: https://www.itntrialshare.org/ACCLAIM_MOA_fig4.url

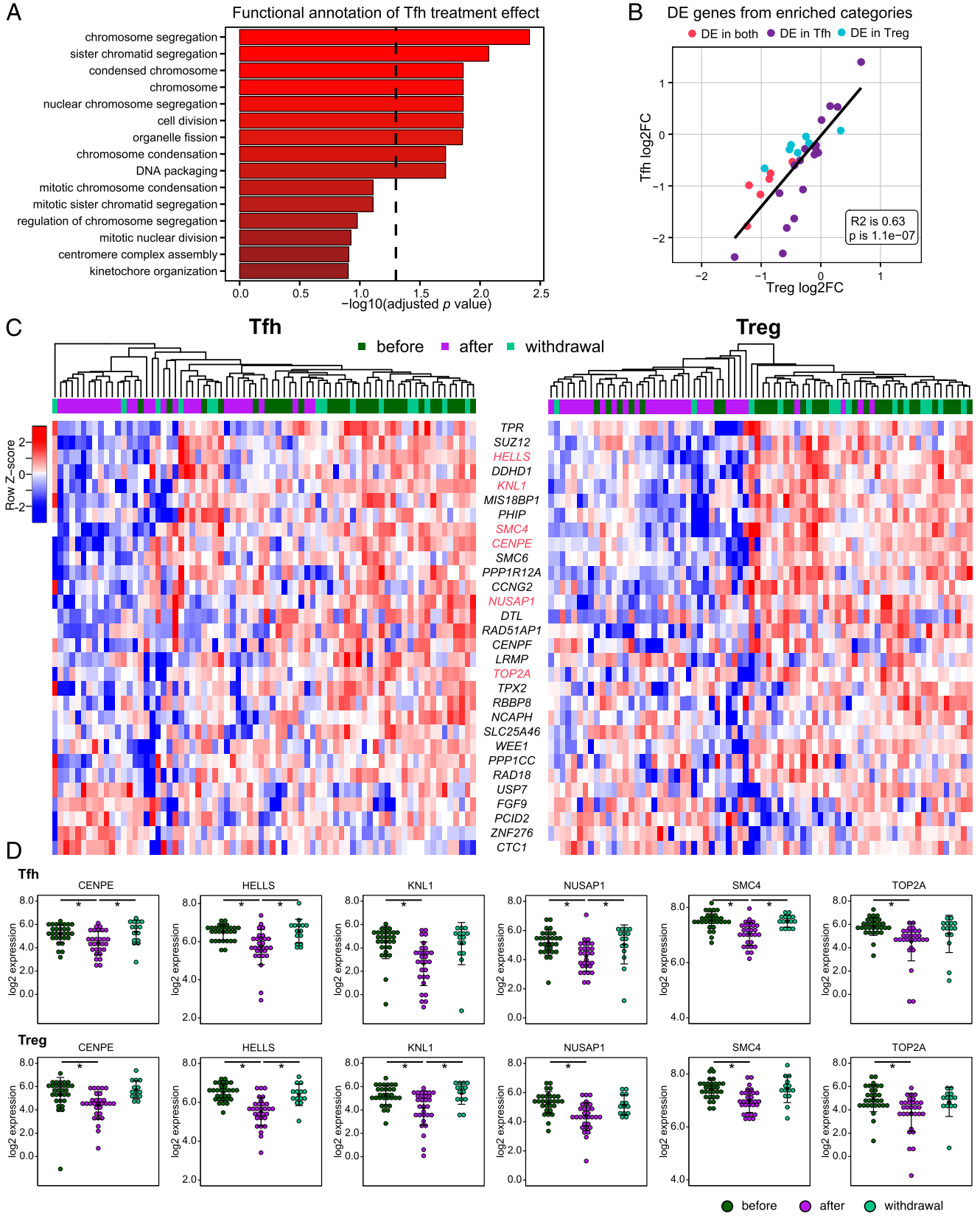

FIGURE 5. DE genes are enriched for genes involved in cell division and chromatin dynamics. (**A**) GO term analysis on DE genes in Tfh. Dashed line represents adjusted p value of 0.05. (**B**) Log2FC of Treg and Tfh cells for genes within significantly enriched GO categories that are DE in at least one of the populations. (**C**) Heatmap of genes plotted in (B). Red gene names indicate that the transcripts were DE upon treatment in both cell populations. (**D**) Normalized log2 expression of genes plotted in (B). Error bars represent one SD. Data for this figure available at: https://www.itntrialshare.org/ACCLAIM_MOA_fig5.url. *Adjusted p value < 0.05.

percentage and activation status of circulating Tfh and plasmablasts after abatacept treatment, we speculate CTLA4-Ig inhibition of CD28 signaling is the dominant mechanism for Tfh maintenance and activation.

The relative contribution of CD28 and ICOS for the generation and maintenance of Tfh cells has been debated. Some studies suggest that Tfh cell differentiation is instructed by ICOS signaling during the priming step (39). However, more recent data support an important role of CD28 signaling early during the generation of Tfh, whereas ICOS signaling may be important later for maintenance of Tfh cells (29, 34, 40–43). Given that abatacept decreased the percentage of circulating Tfh cells and their expression of ICOS, our findings indicate that CD28 signaling may also promote maintenance of Tfh cells, and that the roles of CD28 and ICOS in Tfh cell generation and maintenance may be linked via CD28-dependent ICOS induction (44, 45).

Similar to Tfh cells, CD28 and CTLA4 signaling play complex roles in the development and function of Treg cells. Thymic development and peripheral abundance of Treg cells are substantially reduced in CD28-deficient mice, and consequently, loss of CD28 in prediabetic NOD mice accelerate disease development, whereas transfer of $CD4^+CD25^+$ Treg cells can delay or prevent development of diabetes (46). Interestingly, the function of CTLA4 on Treg cells appears to vary developmentally. Whereas deletion of CTLA4 on Treg cells from birth produces a lethal autoimmune phenotype, inducible deletion of CTLA4 on Treg cells in adult mice increases the abundance and function of Treg cells and renders the animals more resistant to autoimmune disease development (47). Although some studies have proposed that CTLA4-Ig could convert $CD4^+CD25^-$ non-Treg cells into $CD4^+CD25^+$ Treg cells (48), we observed fewer Treg cells in the circulation of RRMS participants treated with abatacept. Thus, as with Tfh cells, we speculate that inhibition of CD28-mediated costimulation appears to be the dominant mechanism of action by which abatacept therapy decreases circulating Treg cells in humans. These results are consistent with studies showing that blockade of CD80/CD86 with CTLA4-Ig leads to a rapid decrease in both thymic and peripheral Treg cells in mice (49, 50) and to a decrease in peripheral Treg cell frequencies in patients with various autoimmune conditions (51). Furthermore, treatment with abatacept specifically altered memory rather than naive Treg cells, which is consistent with studies demonstrating that maintenance of memory Treg cells relies on continued TCR and costimulatory signals (52–54). Importantly, despite the observed reduction in peripheral Treg cells, no evidence of increased disease activity following abatacept treatment was observed either clinically or by magnetic resonance imaging during the clinical trial. However, we cannot exclude the possibility that Treg cell frequencies and/or function in the CNS were not altered by abatacept, or that Treg cell function in either compartment was enhanced by abatacept as previously shown for circulating Treg cells in rheumatoid arthritis patients receiving abatacept therapy and during CTLA4-Ig treatment in vitro (55, 56).

Although we observed a transient decrease in circulating Tfh cells and plasmablasts upon abatacept treatment, the Ag specificity of these cell types and their role in MS pathogenesis is unknown. MS is believed to be a T cell–mediated disease, but evidence including the efficacy of B cell–depleting therapy point to an important role of B cells (57–60). Plasma cells that generate autoantibodies can participate in disease pathogenesis by promoting demyelination (61), whereas B cells that present autoantigens (62) and produce proinflammatory cytokines such as IL-6 (63) can also perpetuate the disease independent of Tfh cells. Therefore, it is possible that Tfh cells and plasmablasts targeted by abatacept do not contribute significantly to disease pathogenesis in the cohort of MS participants from the ACCLAIM trial (RRMS with low disease activity), which could help explain the poor efficacy in this cohort compared with what has been reported in autoimmune conditions strongly associated with aberrant Tfh cell and plasmablast responses (50, 51). Importantly, our results may provide an explanation for why abatacept alone does not promote development of sustained tolerance. First, the effects are transient and not long-lasting, and second, the negative effects on Treg cells may be detrimental to tolerance induction. Modulation of Tfh cells by abatacept may be beneficial in autoimmune diseases in which Abs, B cells, and Tfh cells play a dominant role. However, this beneficial effect should be carefully weighed against the potential detrimental effects of abatacept on $CD45RO^+$ memory Treg cells, especially in autoimmune diseases in which their frequency is limited and/or their function is intrinsically impaired or not effective at regulating pathogenic effector cells, such as MS (64–67).

Acknowledgments

We thank colleagues at the Immune Tolerance Network and the collaborators who contribute in many capacities to Immune Tolerance Network projects and perspectives. We thank the members of the Benaroya Research Institute genomics core for performing the RNA sequencing. The authors are grateful to the ITN035AI ACCLAIM study participants and thank the clinical site investigators and study coordinators. We thank M.K. Kinsman for contributions to the development and performance of flow cytometry assays.

Disclosures

L.A.T. is employed by and holds equity grants in Rheos Medicines. The other authors have no financial conflicts of interest.

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